공학박사 학위논문

Isolation and characterization of *Bacillus* sp. 275 exhibiting lignocellulolytic activities

리그노셀룰로오스 분해 활성

바실러스 275 균주의 분리 및 특성

2017 년 8 월

서울대학교 대학원

공과대학 바이오엔지니어링전공

공 경 택

Abstract

Isolation and characterization of *Bacillus* sp. 275 exhibiting lignocellulolytic activities

Gyeongtaek Gong

Interdisciplinary Program of Bioengineering

The Graduate School

Seoul National University

The objective of this research is to isolate lignocellulose-degrading bacteria from environment and characterize their lignocellulolytic activities. In addition, a study on the lignocellulolytic properties and genomic analysis of selected isolates, *Bacillus* sp. 275 were performed. In this study, various environmental soil samples and isolation media were used to acquire isolates. The cell growth, enzyme activities, and the degradation products of cellulose and xylan were analyzed.

First, bacterial isolation from environmental soils and investigation of their cellulolytic, xylanolytic and lignolytic activities and taxonomic distribution were conducted. Finally, 89 bacterial strains were isolated. Lignocellulolytic bacteria

were isolated from not only lignocellulose-rich soils but also lignocellulose-lack soil samples. All the isolates were included in 4 phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. *Bacillus* and *Streptomyces* occupied over 70 % of bacteria showing multienzymatic activities and the most abundant genus with only lignolytic activity was *Burkholderia*. Lignin-containing medium was effective to isolating bacteria with lignolytic activity only, but not favorable to isolating bacteria exhibiting multienzymatic activities.

Second, candidate bacterial isolate exhibiting high lignocellulolytic activities was selected comparing growth and enzyme activity. *Bacillus* sp. 275 was grown in both solid and liquid alkali lignin media, and showed relatively high cellulase, xylanase and peroxidase activities compared to isolates, strain 123 (i.e., *Bacillus aryabhattai*) and 414 (i.e., *Streptomyces durhamensis*). As a result, *Bacillus* sp. 275 was selected as final candidate for degradation of lignocellulose among 89 isolates.

Third, whole genome sequencing of *Bacillus* sp. 275 was conducted and genomic analysis using complete genome sequence was investigated. A lot of genes concerning to the degradation of lignocellulose in the genome of *Bacillus* sp. 275 were detected. Endoglucanase, β-glucanase, glucohydrolase, glucosidase, 1,4-β-xylosidase, arabinoxylan arabinofuranohydrolase, glucuronoxylanase, dye decolorizing peroxidase and laccase were present in the genome of *Bacillus* sp. 275. Besides, the genes related to degradation of starch, mannan, galactoside and arabinan were also found.

Finally, the characteristics of lignocellulolytic properties of *Bacillus* sp. 275 were investigated. The degradation products of cellulose (i.e., cellobiose and cellotriose) and xylan (i.e., xylose, xylobiose, xylotriose, xylotetraose and

xylopentaose) were observed by extracellular proteins secreted by *Bacillus* sp. 275. In addition, filter paper degradation occurred actively by *Bacillus* sp. 275 for 11 days. Regarding lignin utilization, the cell growth with alkali lignin added to the culture medium (LB medium) was increased by 78% compared to that without alkali lignin. Moreover, the lag phase was shortened from 12 hours to 6 hours in the case of lignin-added culture medium.

In this research, the isolation condition such as the sampling locations and the isolation medium was significant factors to efficiently isolating bacteria with lignocellulolytic activities. *Bacillus* sp. 275 showed cellulolytic, xylanolytic and lignolytic activities by extracellularly produced enzymes. In addition, *Bacillus* sp. 275 is potentially capable of degrading wide ranges of polysaccharides in lignocellulose. Considering only a few reports of bacteria using all major three component of lignocellulose (i.e., cellulose, hemicellulose and lignin) were published, *Bacillus* sp. 275 is a promising candidate for degrading or utilizing lignocellulosic biomass.

Keywords: bacterial isolation, lignocellulolytic bacteria, lignocellulose degradation, *Bacillus* sp. 275, complete genome sequencing

Student Number: 2008-30288

Contents

List of Tables

List of Figures

Chapter 1.

Research background and objective

Chapter 1. Research background and objective

The use of lignocellulosic biomass is important and promising strategy for a sustainable development inhibiting the problems of fossil resources. In addition, lignocellulosic biomass does not compete with edible biomass such as corn, wheat, sugar cane and sugar beet, and has lower cost than food crops[1]. In spite of these advantages, efficient utilization of lignocellulosic biomass is limited because of the natural structure (i.e., cross-link in polysaccharides with lignin via ether and ester bonds[2]) of lignocellulose.

In nature, microbial degradation of lignocellulose is carried out by microorganisms, which have distinct enzymatic systems for degradation of polysaccharides (e.g., cellulose and hemicellulose) and lignin. These cell wall-deconstructing enzymes are unusually classified diversely such as glycoside hydrolases, polysaccharide lyases, carbohydrate esterase and auxiliary activities[3]. Therefore, the isolation of bacteria which have high lignocellulolytic activities is significant for utilization of lignocellulose. Despite the vast efforts underway to isolate lignocellulose-degrading bacteria, the use of various environmental sources and isolation media to explore taxonomic distributions of lignocellulolytic bacteria and the related lignocellulolytic activities has not been reported yet. The isolation conditions are important factors in the acquisition of efficient lignocellulose degraders.

Currently, studies for degradation of lignocellulose have mainly focused on degradation of cellulose, hemicellulose (or xylan) or lignin, separately. However, it is necessary to use all the main three compositions of lignocellulose.

In this research, isolation of lignocellulolytic bacteria was conducted and their cellulolytic, xylanolytic and lignolytic activities were analyzed on solid culture media. Among 89 isolates from environmental soil samples, *Bacillus* sp. 275 was selected as a candidate with multienzymatic activities for lignocellulose degradation. *Bacillus* sp. 275 has genes encoding lignocellulolytic enzymes according to the information of complete genome and also has the cellulolytic, xylanolytic and lignolytic enzyme activities. The characteristics of *Bacillus* sp. 275 provide insight into bacterial degradation of all main components in lignocellulosic biomass.

In summary, the objectives of this thesis are as follows:

1. Isolation and characterization of cellulolytic, xylanolytic and lignolytic bacteria from environmental sources
2. Selection of candidate bacterial isolate exhibiting high lignocellulolytic activities
3. Analysis of lignocellulose-degrading genes using complete genome sequence of *Bacillus* sp. 275
4. Investigation into characteristics of lignocellulolytic properties of *Bacillus* sp. 275

This thesis provides previously unknown insight into the sampling points and isolation medium for efficient isolation of lignocellulose-degraders. In addition, *Bacillus* sp. 275 has potentially wide range of use in the degradation of polysaccharide and lignin in lignocellulosic biomasses. Therefore, *Bacillus* sp. 275 can be useful microorganism for conversion of lignocellulose into useful chemicals and fuels.

Chapter 2.

Literature review

Chapter 2. Literature review

2.1. Lignocellulosic biomass

Lignocellulosic biomass is considered the most abundant[4-6] and renewable[7, 8] resources on earth. Moreover, lignocellulosic biomass does not interfere with food biomass and not threat to food security[9]. Lignocellulose is the major component of plant cell walls and different lignocellulosic biomasses such as wood, switch grass and agricultural residues can be used for biofuels or chemicals.

Lignocellulose is composed of three major components which were cellulose, hemicellulose and lignin[8]. These macromolecules construct a complex structure by aromatic heteropolymer (e.g., lignin) and help resistant from environmental attacks by three-dimensional structures. The ratio of cellulose, hemicellulose and lignin is various depending on the characteristic of the biomass. The composition of various lignocellulosic biomasses was summarized in Table 2.1.1[10].

2.1.1. Cellulose

Cellulose is the main component of lignocellulose and a homo-polymer of glucose linked by beta(1-4) linkages[4]. In general, the proportion of cellulose is 15 ~ 50%[11] of the dry weight of biomass. Cellulose is difficult for degradation to glucose because of its crystalline structure[12, 13]. In contrast, amorphous cellulose is more easily attacked by enzymes[14]. Cellulose fibers are used in the paper industry.

Table 2.1.1 Composition of various lignocellulosic biomasses[10]

Lignocellulosic biomass	**Cellulose[1)]**	**Hemicellulose[1)]**	**Lignin[1)]**
Agricultural residues	37-50	25-50	5-15
Bagasse	54.87	16.52	23.33
Barley hull	34	36	19
Barley straw	36-43	24-33	6.3-9.8
Corn straw	42.6	21.3	8.2
Corn cobs	45	35	15
Corn stover	38	26	19
Douglas fir	35-48	20-22	15-21
Grasses	25-40	25-50	10-30
Hardwood	40-55	24-40	18-25
Newspaper	40-55	25-40	18-30
Oat straw	31-35	20-26	10-15
Poplar wood	45-51	25-28	10-21
Pine	42-49	13-25	23-29
Rice Straw	32.1	24	18
Rice husk	28.7-35.6	11.96-29.3	15.4-20
Sugar cane bagasse	42	25	20
Softwood	45-50	25-35	25-35
Wheat straw	29-35	26-32	16-21
Wheat bran	10.5-14.8	35.5-39.2	8.3-12.5

1) Dry weight percentage

2.1.2. Hemicellulose

Hemicellulose is hetero-polysaccharides composed of pentoses such as xylose and arabinose, hexoses such as glucose, mannose and galactose, and acetylated sugars. The composition and structure are different according to the lignocellulosic materials[4]. For example, O-Acetyl-4-O-methylglucuronoxylans in hardwoods constitute 10–35% of the hemicelluloses, and arabino-4-O-methylglucuronoxylans in softwoods constitute 10–15 % of the hemicelluloses.

However, xylan (the polymer of xylose) is the major component of hemicellulose up to 50% and the second most abundant polysaccharide after cellulose[14, 15]. A various linkages such as alpha-1,2, alpha-1,3 are used for connect to the other sugars. These complex structures provide a barrier from microbial and mechanical forces. To link hemicellulose and cellulose, hydrogen and covalent bonding is used. Deconstruction of hemicellulose is easier than cellulose because of a low molecular weight of hemicellulose.

2.1.3. Lignin

Lignin is an amorphous highly branched aromatic polymer made by the random coupling with three types of monolignols (i.e., coumaryl, coniferyl and sinapyl alcohols)[4]. These monolignols are linked by β-aryl ether, di-aryl propane, biphenyl, diaryl ether, phenylcoumarane, spirodienone and pinoresinol bonds[14]. The proportion of lignin is 15 ~ 40% of the dry weight of biomass[16]. However, the composition of lignin is different. In general, hardwoods are composed of 56%

coniferyl alcohols and 40 % sinapyl alcohols while softwood is mainly composed of coniferyl alcohols (80 %)[17]. Table 2.1.2 shows the typical linkages of lignin in softwood and hardwood[18]. Lignin is difficult to breakdown because of its complex chemical nature (protecting cellulose and hemicellulose polymers from the external attack by other microbes)[10, 19]. Because of the difficult use of lignin, it is mainly burned as a source of energy in the pulp and paper industry[8]. Lignin also used as adhesives, food additives and production of phenolic compounds[14]. In addition, lignin and lignin derivatives can be used to plastics and resins as biopolymers[20, 21]. However, recalcitrant to degradation, lignin does not increase in nature.

2.2. Microbial degradation and utilization of Lignocellulosic biomass

Although lignocellulose is an abundant and available renewable resource, utilization of lignocellulose as substrates by microbial cells has been known as one of the most difficult processes[22]. Because of difficulty from complex substrates and diversity of various enzymes, microbial community or consortia were also used for lignocellulose degradation[23-28]. Moreover, a fungal-bacterial microbial consortium was used for enhancement of lignocellulolytic activity[29].

However, the major research for bacterial degradation of lignocellulose is focused on single bacterial strain.

2.2.1. Cellulose degradation

Table 2.1.2 Typical linkages of lignin in soft wood and hard wood[18]

Linkages	**Softwood (%)**	**Hardwood (%)**
β -O-4	~50	60
5-5	9.5-11	6-8
β -5	9-12	6
β -1	7	7
α -O-4	6-8	6-8
4-O-5	3.5-4	6.5
β -β	2	3

Cellulose was typically degraded by three types of enzymes: endoglucanase, cellobiohydrolase and beta-glucosidase[30]. Endoglucanase break the internal bonds of the amorphous regions of cellulose polymer. Cellobiohydrolases hydrolyze β (1–4)-d- glucosidic linkages in cellulose, generating cellobiose from either the reducing or the non-reducing ends of the polymer. Next, β-glucosidases degrade the disaccharide cellobiose to glucose. Recently, lytic polysaccharide monooxygenases (LPMOs) can be oxidized cellulose[12, 31-35]. These enzymes are disrupted the cellulose fiber structure, thereby motivating a release of elementary nonofibrils. Therefore, cellulase can be degraded more efficiently by oxidative cleavages of LPMOs.

2.2.2. Hemicellulose and xylan degradation

As the same as cellulose degradation, the enzymatic breakdown of hemicellulose needs an consortium of various enzymes such as endo-xylanase, beta-xylosidase, 1,2-alpha-glucuronosidase, beta-galactosidase and etc[36]. The degradation of xylan, the major component of hemicellulose, the existence of enzymes which break backbone and various side-chains is essential[15]. Xylanase is one of the major enzyme related to hemicellulose degradation, which cleaves the beta-1,4-linked D-xylopyranose units from the backbone structure of xylan. Xylanase is widely distributed in bacteria, fungi, plants and insects. According to the carbohydrate-active enzymes (CAZy) database (http:/www.cazy.org), more than six glycoside hydrolases (GH) families (e.g., 5, 7, 8, 10, 11, 26, 30 and 43) are related to xylanase. Among various glycoside hydrolases families, xylanases from

GH10 and GH11 families are widely studied. Xylanase from GH11 family are only acted to xylan, compared to xylanase from GH10 are functioned to not only xylan but also substituted linkages of the xylan backbone[15]. The dominant studies about optimum condition for xylanases are mesophilic temperature and neutral or some acidic pH. However, xylanases which are active at 5~105°C, pH from 2 to 11 and NaCl concentrations as high as 30% have been reported[37].

2.2.3. Lignin degradation

Enzymatic degradation of lignin is involved in enzymes of oxidative reaction. The famous enzymes of lignin degradation were laccase and peroxidases such as lignin peroxidase, manganese peroxidase, versatile peroxidase and dye-decolorizing peroxidase. Lignin peroxidase degrades through a mechanism based on the formation of radicals in the presence of hydrogen peroxide. Lignin peroxidase is capable of cleaving alkyl side chains in lignin and lignin model compounds. Manganese peroxidase uses Mn^{2+} as an electron donor which is oxidized to Mn^{3+} through peroxide dependent oxidation. Manganese peroxidase is considered a familiar mechanism for the degradation of non-phenolic structures. Versatile peroxidase can also oxidize Mn^{2+} and non-phenolic lignin model compounds like manganese peroxidase. However, the versatility of versatile peroxidase could be possible for oxidation of both low and high redox potential aromatic substrates. Dye-decolorizing peroxidase is a new superfamily of heme-containing peroxidase which was found in both bacteria and fungi[38]. The catalytic cycle is not well-known yet. Laccase is a multicopper-containing enzyme with

oxygen as an electron acceptor. In general, laccase can oxidize the wide spectrum of phenolic compounds[18]. Recently, superoxide dismutase is considered as a candidate for degradation of lignin[39, 40].

Among various kinds of bacteria, lignin degradation using *Rhodococcus*[41-47], *Pseudomonas*[48-54] and *Streptomyces*[55-63] genus were widely studied. *R. jostii* RHA1 is a famous strain degrading polychlorinated biphenyl and lignin. 96 mg/L of vanillin[46] and 80-125 mg/L of pyridine 2, 4-dicarboxylic acid (2, 4-PDCA) or pyridine 2,5-dicarboxylic acid (2,5-PDCA) were produced by these strain. In addition, DyP-type peroxidase in *R. jostii* RHA1 is related to lignin degradation by beta-aryl ether cleavage[64]. *R. opacus* DSM 1069 and PD630 were also studied about production of lipid from lignin[65, 66]. *Pseudomonas* sp. PKE117[53, 54], *P. fluorescens* Pf-5[50], *P. putida* KT2440[48] and MET94[51] were reported degradation of lignin and pretreated lignocellulosic biomass in *Pseudomonas* genus. Especially, *P. putida* KT2440 is the most studied strain in this genus. This strain can be produced bioplastics and polyhydroxalkanate (PHA). More than 55% of cell dry weight of mutant of *P. putida* was PHA[67]. Among *Streptomyces* genus, *Streptomyces viridosporus* T7A is one of the most well studied. *S. viridosporus* T7A can degrade wide range of lignin (e.g., softwood, hardwood and grass lignin) and secret the ligninolytic enzymes such as lignin peroxidase and laccase[62, 68-70]. Some *Bacillus* species are reported as lignin or lignin analogue degraders[39, 71-75]. Besides these bacteria, *Pandoraea* sp. B-6[76], *Novosphingobium* sp. B-7[77] and *Pandoraea* sp. ISTKB[78] can be degraded kraft lignin as a sole carbon source. *Enterobacter lignolyticus* SCF1[79, 80] and *Burkholderia* sp. LIG30[81] are also known as lignin-degrading bacteria.

Table 2.2.1 Summary of bacterial enzymes for lignin degradation

Enzyme	Bacterium	Reference
DypB	*Rhodococcus jostii* RHA1 *Pseudomonas fluorescens*	Ahmad *et al.*[44] Rahmanpour *et al.*[50]
Dyp2	*Amycolatopsis* sp. 75iv2	Brown *et al.*[82]
Laccase	*Streptomyces coelicolor*	Majumdar *et al.*[69]
CopA	*Pseudomonas stutzeri*	Strachan *et al.*[83]
Etherase	*Sphingobium* SYK6 *Novosphingobium* sp.	Gall *et al.*[84] Picart *et al.*[85]

2.2.4. Degradation for multicomponent of lignocellulose

Some bacteria can degrade multicomponent of lignocellulosic biomass such as cellulose and hemicellulose (or xylan), cellulose and lignin, hemicellulose and lignin, or all components of lignocellulose. The isolates named as *Bacillus* sp. CS-1 was decomposed cellulose, hemicellulose and lignin in rice straw[72].

Similarly, the combination of cellulase, hemicellulase and ligninase is studied for increase of degradation yield. Synergistic interaction of cellulase and xylanase was detected on unpretereated lignocellulosic substrates. The hydrolysis yields of lignocellulose treated by cellulase and xylanase mixture was improved by 133-545% than only treated with cellulase[86]. Similarly, the synergistic effect of cellulase and xylanase enzymes was shown by improvement of cellulose accessibility[87]. Moreover, cellulase, xylanase and laccase in the designed system (e.g., designer cellulosome) were enhanced decomposition of lignocellulose[88].

2.3. Screening and isolation of lignocellulolytic bacteria

There are two screening methods for isolation of ligninolytic prokaryotes: culture-dependent and culture-independent techniques[89]. Culture-dependent screening method detects bacterial growth or utilization of natural lignin, lignin model compounds[90] or aromatic dyes. In contrast to culture-dependent method, culture-independent screening use genetic information such as metagenomics, metatranscriptomics and bioinformatics[83, 91-96].

Vanillin is regarded one of the major valuable products from lignin[97]. The

vanillin-sensing cell was developed in *E. coli*[98] that did not show cross-activity of lignin, lignin degradation products and vanillin analogues. High-throughput screening of lignin-degrading enzymes (i.e., vanillin producing enzymes) is possible by this microorganism. In addition, screening of bacterial isolate capable of producing vanillin and vanillic acid was developed by the colorimetric method using ferulic acid as a sole carbon source[99].

2.4. Lignin degradation pathway

From various lignin degradation pathways, beta-aryl ether and biphenyl catabolic pathways are important because of highly dominant linkages in lignin (e.g., β-O-4, 5-5). Through these pathways, one of the valuable products likes vanillin or 4-hyddroxybenzoic acid was produced.

Degradation pathway of beta-aryl ether has been reported mainly in *Sphingobium* sp. SYK-6[100-102]. Dehydrogenase (LigD), beta-etherase (LigE and LigF) and other enzymes such as LigG, LigL, LigN and LigO were participated in the degradation of beta-aryl ether linkage. Regarding biphenyl degradation pathway, extensive studies were conducted because of polychlorinated biphenyl which is a major pollutant in environment. Biphenyl 2,3-dioxygenase, LigX, LigZ, LigY, LigW and LigW2 were associated with this pathway.

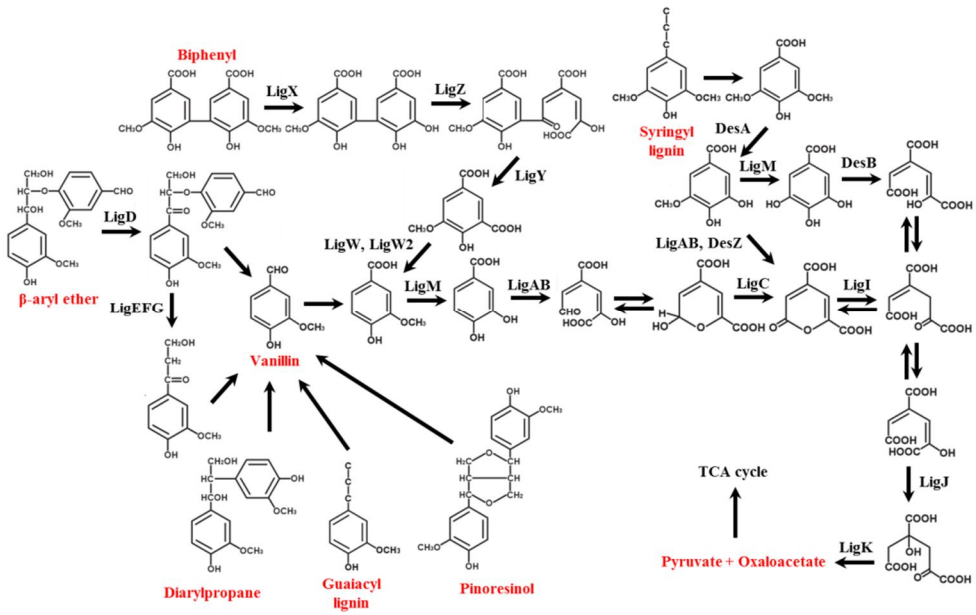


Figure 2.4.1 Catabolic pathways for the degradation of lignin-derived aromatic compounds by *Sphingobium* sp. SYK-6[103].

Chapter 3.

Experimental procedures

3.1. Acquisition of environmental samples

Environmental soil samples were collected from mountains, a wetland and mudflat. Eight soil samples were collected including 5 soil samples from 4 mountains, 2 samples from a wetland and 1 sample from a mudflat. The mountain soil samples were taken from under rotten wood at a depth of 20-30 cm. The wetland soil was taken from the boundary between fresh water and land at a depth of 10-20 cm. The mudflat soil was taken at a depth of 10-20 cm during the ebb tide. The soil samples were used for bacterial isolation within 3-5 hours of collection.

3.2. Isolation of bacteria with potential lignocellulolytic activities

One gram of each environmental sample was suspended in 10 mL of 0.9% sodium chloride solution and the suspension was used as an inoculum for agar plates to isolate lignocellulolytic microorganisms. The isolation medium contained (in 1 L of distilled water) 0.3 g of NH_4Cl, 0.3 g of $MgCl_2$, 0.3 g of $CaCl_2$, 0.3 g of KH_2PO_4, 0.5 g of yeast extract, 10 mL minimal trace element solution (DSM medium 318) and 5 g of carboxymethyl cellulose (CMC, soluble in water) or 5 g of microcrystalline cellulose (CRY, insoluble in water) as a carbon source. Additionally, instead of cellulose, 2 (cellulose and xylan) or 3 (cellulose, xylan and lignin) components of lignocellulose at a total concentration of 5 g/L were used as carbon sources: 2.5 g/L of CMC + 2.5 g/L of xylan (CX), 1.7 g/L of CMC + 1.7 g/L

of xylan + 1.6 g/L of alkali lignin (CXL). To prepare agar plates, agar powder was added at a final concentration of 15 g/L.

Isolating bacteria with potential lignocellulolytic activities was performed by directly spreading each suspended environmental sample (0.2 mL) on the isolation medium agar plate (CMC, CRY, CX and CXL) after serial dilution of 10^{-4} ~ 10^{-7} with 0.9% sodium chloride solution. Morphologically different colonies on the agar plates were transferred to fresh agar medium 3-4 times until acquiring a single bacterial colony. All the agar plates were incubated at 30°C until a colony appeared and then stored at 4°C prior to use.

3.3. Screening and estimating cellulolytic, xylanolytic and lignolytic activities on agar plates

Cellulase and xylanase activities were determined with the agar medium containing 5 g/L of CMC or xylan as a carbon source using the Congo red staining method to detect the clear zone around the colony[104]. To estimate cellulolytic and xylanolytic activities, a single colony of each isolate from agar plate was steaked onto CMC or xylan agar plate. When the colony appeared after 5 days of incubation on CMC or xylan agar plates, 0.1% of Congo red solution was flooded on the agar plate for 15 minutes. Then, the agar plate was washed with 1M NaCl solution for 15 minutes. The enzyme activity was calculated by the "Ratio of relative enzyme activity, I_{CMC} or I_{xylan}=diameter of halo zone/diameter of colony" [105]. The intensity of cellulolytic and xylanolytic activities was classified into four

groups: strong (+++: Indices of relative enzyme activity was more than 10), medium (++: Indices of relative enzyme activity was between 10 and 3), weak (+: Indices of relative enzyme activity was below 3) and none (no halo zone detected).

To screen for a lignin degradation ability, a single colony of each isolate was picked and streaked onto an agar plate containing a lignin analogue, Azure B (0.01 w/v %) with 1 g/L of glucose and xylose as carbon sources. After 7 days of incubation at 30°C, the lignolytic activity was detected by observing a decolorizing zone around a colony. Additionally, the capability of lignin utilization was screened by growth on alkali lignin agar plate without any supplemented carbon sources and yeast extract. The growth of the isolate was also compared with the growth on a plate deficient in alkali lignin. After incubating 7 days at 30°C, colony formation and size were compared between the lignin agar plate and the agar plate with no carbon source (lignin-deficient agar plate). The intensity of the lignolytic activity was classified into four groups: strong (+++: Azure B decolorization and growth on the lignin agar plate compared with the lignin-deficient agar plate), medium (++: no decolorization of Azure B and growth on the lignin agar plate compared with the lignin-deficient agar plate) , weak (+:decolorization of Azure B and no growth on the lignin agar plate compared with the lignin-deficient agar plate) and none (no decolorization of Azure B and no growth on the lignin agar plate compared with the lignin-deficient agar plate).

3.4. Identification of isolate with 16S rRNA gene analysis

The genomic DNA of the isolate was extracted with a G-spin genomic DNA

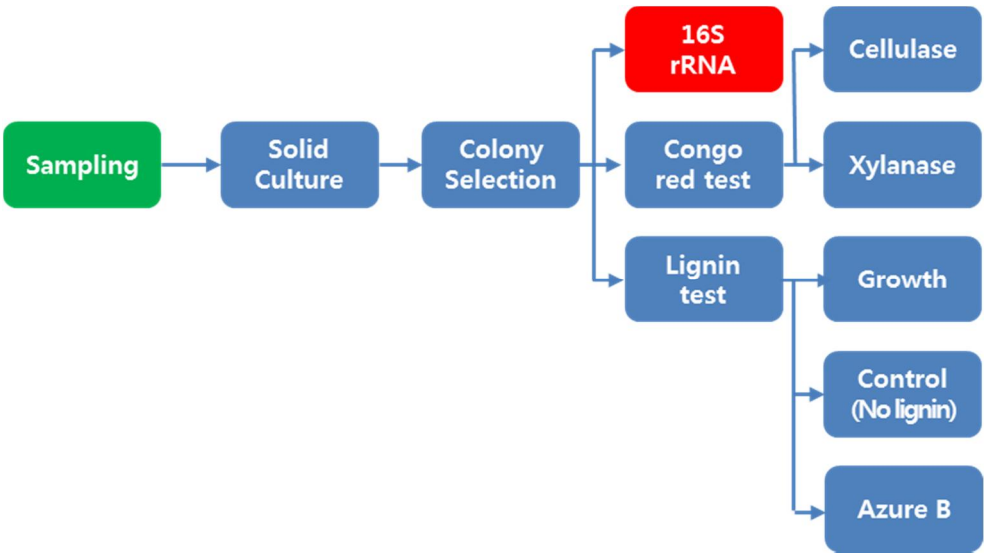


Figure 3.3.1 Experimental flowchart of a process for isolating, screening and estimating cellulolytic, xylanolytic and lignolytic activities on agar plates.

extraction kit (Intron, Republic of Korea) following the manufacturer's instructions. The 16S rRNA gene was amplified using AccuPower PCR PreMix kit (Bioneer, Republic of Korea) and the primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The sequence of 16S rRNA was analyzed by Macrogen Inc. (Republic of Korea). The isolates were identified using the EzTaxon server (http://www.ezbiocloud.net[106]) based on the 16S rRNA sequence data. Phylogenetic tree was generated by the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA7[107] and the distances were computed using the Maximum Composite Likelihood method.

3.5. Selection of high lignocellulolytic bacteria

3.5.1. Cellulolytic, xylanolytic and lignolytic activities measurements of cell culture supernatant of the selected isolates

The isolates were pre-cultured by inoculated single colony in 2 mL of LB medium containing 10 g/L of tryptone, 5 g/L of yeast extract and 5 g/L of NaCl at 30°C on a shaking incubator at 200 rpm. After one day incubation, 1% of cell broth was inoculated in 20 mL of LB medium in 150 mL straight neck flask for other one day. Culture supernatant was acquired by centrifuged at 14,000×g for 5 min of cell broth. Protein concentration was measured by Bradford protein assay reagent (Bio-Rad) and Cellulase (endo-cellulase) activity was quantification by Cellulase assay kit (CELLG5 method) (Megazyme). Xylanase activity was measured with xylan as

substrate and detected by 3,5-dinitrosalicylic acid (DNS) colorimetric method at 50°C. Peroxidase and laccase activities was assayed by detecting oxidation of ABTS (ε_{420nm} = 3.6 mM^{-1} cm^{-1}) in 100 mM acetate buffer at pH 3 and room temperature with or without H_2O_2, respectively. Peroxidase activity was calculated by differences between two measuring conditions. One unit of enzyme activity was defined as the amount of enzyme required to release one micromole of product per minute.

3.5.2. Liquid culture in alkali lignin and mixed carbon medium consisting of cellulose, xylan and lignin

Ten selected isolates were pre-cultured in 2 mL of isolation medium contained (in 1 L of distilled water) 0.3 g of NH_4Cl, 0.3 g of $MgCl_2$, 0.3 g of $CaCl_2$, 0.3 g of KH_2PO_4, 0.5 g of yeast extract, 10 mL minimal trace element solution (DSM medium 318). After one day incubation, the supernatant was removed by centrifugation. The cell pellet was resuspened with isolation medium except yeast extract. Cell broth was inoculated in isolation medium except yeast extract with CXL (1.7 g/L of CMC + 1.7 g/L of xylan + 1.6 g/L of alkali lignin) and 5 g/L of alkali lignin (LGN) as a sole carbon sources, respectively.

3.5.3. Analysis of cellulase, xylanase and peroxidase

The composition of CXL and LGN media was the same as described in

section 3.5.2. The composition of CMC and XYL media were the same as CXL and LGN media except the carbon sources changed to CMC and xylan.

The peroxidase activity was measured by oxidation of 2,4-dichlorophenol (2,4-DCP). The supernatants of cell broth was reacted with a mixture of 50 mM of potassium phosphate buffer (pH 7.0), 3 mM 2,4-DCP, 0.164 mM aminoantipyrine and 4.0 mM hydrogen peroxide. After 1 hour of incubation at 37°C, the different of absorbance at 510 nm in the reaction mixture was detected. The cellulase and xylanase activities were detected using DNS methods as mentioned in section 3.5.1.

3.5.4. Oxidation of veratryl alcohol

2.0 mM of veratryl alcohol and 0.5 mL of the supernatant were mixed, and then added of 0.4 mM of hydrogen peroxide in total volume of 1 mL (pH 3.0 and 30°C). The product of oxidation of veratryl alcohol (e.g., veratryl aldehyde) can detected at 310 nm using UV spectrophotometer.

3.6. Genome sequencing

The genome of *Bacillus* sp. 275 was sequenced using a combination of the PacBio RSII system (Pacific Biosciences, CA, USA), Illumina MiSeq (100-bp paired-end) and the Roche 454 sequencing TITAN technology. All the reads were assembled using the Roche gsAssembler 2.6, CLC Genomics Workbench 6.5.1 and

Table 3.5.1 Measurements of enzyme activities in cell culture supernatant

Enzyme	**Method**
Cellulase	Cellulase assay kit (CELLG5)
Xylanase	3,5-dinitrosalicylic acid (DNS) colorimetric method
Laccase	ABTS oxidation
Peroxidase	ABTS oxidation 2,4-dichlorophenol (2,4-DCP) oxidation veratryl alcohol oxidation

PacBio SMRT Analysis 2.1 with a genome coverage of 276 folds.

3.7. Degradation of lignocellulose by *Bacillus* sp. 275

3.7.1. Cell cultures in complex and defined media

The cell growth in complex medium was studied in 20 mL of LB medium in 150 mL straight neck flask. Alkali lignin was added from 2% stock solution to final concentration of alkali lignin at 0.05% and 0.1%.

The defined medium based on M9 was composed of 12.8 g/L of $Na_2HPO_4 \cdot 7H_2O$, 3.0 g/L of KH_2PO_4, 10.0 g/L of NaCl, 1.0 g/L of NH_4Cl, 0.492 g/L of $MgSO_4 \cdot 7H_2O$, 0.111 g/L of $CaCl_2$ and trace element (0.1 mg/L of $MnCl_2 \cdot 4H_2O$, 0.07 mg/L of $ZnCl_2$, 0.06 mg/L of H_3BO_3, 0.04 mg/L of $NaMoO_4 \cdot 2H_2O$, 0.2 mg/L of $CoCl_2 \cdot 6H_2O$, 0.02 mg/L of $NiCl_2 \cdot 6H_2O$, 0.02 mg/L of $CuCl_2 \cdot 2H_2O$, 2.78 mg/L of $FeSO_4 \cdot 7H_2O$ and 0.1 ul/L of HCl). The carbon source was glucose (3 g/L) and the final concentration of added lignin was 0.05%. Glucose concentration was detected by reflectometer Merck RQflex plus 10 meter using glucose test strips.

3.7.2. Gel permeation chromatography (GPC)

The distribution of molecular weight of lignin was analyzed by GPC on HPLC (Agilent) with a Photodiode Array (PDA) detector and TSK-GEL W3000G column (300 x 7.5 mm). 50 mM NaOH containing 20% acetonitrile was used as eluent. The

eluent flow rate was set to 0.6 mL/min.

3.7.3. Thin layer chromatography (TLC)

The hydrolysis products of cellulose and xylan were analyzed by thin layer chromatography (TLC) on Silica Gel 60 plates (Merck). One microliter of samples was spotted on plate, and dried at room temperature around 5 min. The mixture of 63 ml of 2-propanol, 16 mL of 1-butanol and 21 mL of water was used as a developing solution (100 mL total). After developing, a visualization solution (10% sulfuric acid in ethanol) was sprayed using TLC sprayer. The products were visualized by heating with heat gun.

3.7.4. High performance liquid chromatography (HPLC)

The sugars released by cellulose and xylan were detected using high performance liquid chromatography (Agilent HPLC, USA) equipped with an Aminex HPX-87P column [300×7.8mm] (Bio-rad, USA). The column temperature was 80°C and flow rate of the mobile phase (water for HPLC) was 0.15 mL/min.

3.7.5. Cell cultures in xylan-based media

The composition of a xylan-based medium was based on M9 medium

as mentioned in section 3.7.1. The difference was the concentration and combination of carbon sources. Three kinds of medium composition were 5.0 g/L of xylan (X), 5.0 g/L of xylan + 5.0 g/L of CMC (CX) and 5.0 g/L of xylan + 5.0 g/L of CMC + 0.5 g/L of alkali lignin (CXL).

Cellulase and xylanase activities were measured CELLG5 method and DNS method, respectively, as described in section 3.5.1.

3.7.6. Filter paper degradation

Filter paper degradation was conducted by supplemented with Whatman no. 1 filter paper (1 × 4 cm strip × 4ea) in 5.0 g/L of xylan + 5.0 g/L of CMC (CX) medium as mentioned in section 3.7.5.

Chapter 4.

Isolation of cellulolytic, xylanolytic and ligninolytic bacteria from various environmental sites

4.1. Introduction

Recently, diverse lignocellulolytic bacterial strains have been isolated from environmental sources such as tropical forest soil[108] and wood chips[109] in which natural degradation of lignocellulose was occurring. In addition, taxonomic characterization of lignocellulolytic bacteria and enzymatic activities has been investigated. Industrial lignocellulose streams including black liquor, paper mill effluent and bamboo slips[110, 111] have been also tried as environmental sources to enrich lignocellulolytic bacteria. Likewise, most previous reports for isolation of lignocellulolytic bacteria have been conducted using lignocellulose-abundant or lignocellulose-contained environmental sources[112-114] and the solid agar media containing a single lignocellulose component (cellulose, xylan or lignin) to assay lignocellulolytic activity. However, despite the vast efforts underway to isolate lignocellulose-degrading bacteria, use of various environmental sources and isolation media to explore taxonomic distributions of lignocellulolytic bacteria and the related lignocellulolytic activities has not been reported yet. Because the degradation of lignocellulose is the first key step in lignocellulose utilization, special attention is necessary to screen lignocellulose-degrading bacteria and enzymes with effective methodology.

In this work, the isolation and taxonomic identification of cellulolytic, xylanolytic and lignolytic bacteria were performed using: i) various environmental sources including lignocellulose-rich environmental samples (mountain forest soils near rotten wood) and lignocellulose-rare samples (mudflat soil with a high salt concentration and wetland soil with freshwater) and ii) the isolation medium with a

combination of cellulose, xylan and lignin. In addition, the bacterial taxonomic distribution of the isolates was analyzed with respect to environmental sources and isolation media. This study shows the importance of isolation conditions such as environmental sampling locations and isolation media to effectively obtain lignocellulolytic bacteria.

4.2. Overall taxonomic distribution of isolated bacterial strains

For the isolation of lignocellulose-degrading bacteria, 8 sampling points were chosen from various environmental conditions: 5 mountain soils under rotten wood, 2 wetland samples and 1 mudflat sample (Table 4.2.1). Colonies on the agar plates with CMC, CRY, CX and CXL media were selected based on different morphological characteristics (e.g., size, shape and color). Among 124 isolates, 18 isolates could not be identified by 16S rRNA gene analysis because of either indecipherable overlapping chromatograms or PCR failure. Additionally, 17 isolates from CMC (2 isolates), CRY (4 isolates), CX (3 isolates) and CXL (8 isolates) media were excluded for further study because neither cellulolytic, xylanolytic nor lignolytic activity was shown on the agar plates. Therefore, 89 isolates were used for the 16S rRNA sequence-based taxonomic analysis and assay of the bacterial lignocellulolytic properties.

Table 4.2.2 shows the taxonomic distribution of the 89 isolates. All the isolates were included in 4 phyla, 6 classes, 9 orders, 10 families and 20 genera. Most of

the isolates (98%) belonged to 3 phyla: *Firmicutes* (46%), *Proteobacteria* (37%) and *Actinobacteria* (15%). *Bacteroidetes* accounted for only 2% of the total isolates. Interestingly, only a single class, order and family were observed with the phyla *Actinobacteria, Bacteroidetes* and *Firmicutes*. Moreover, only a single genus, *Bacillus*, was identified in *Firmicutes*. Notably, *Bacillaceae* and *Bacillus* were the most dominant family and genus, respectively, comprising up to 46% of the total isolates affiliated to *B. aryabhattai* (10 isolates), *B. anthracis* (7 isolates), *B. thuringiensis* (7 isolates), *B. acidiceler* (6 isolates) and other *Bacillus* species (11 isolates). Unlike the isolates belonging to *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, 33 isolates in *Proteobacteria* were distributed over 3 classes, 6 orders, 7 families and 14 genera. Overall, in the order of abundance, the most dominant family was *Bacillaceae* (41 isolates, 46%) followed by *Streptomycetaceae* (13 isolates, 15%), *Burkholderiaceae* (10 isolates, 11%) and *Enterobacteriaceae* (8 isolates, 9%).

Four phyla (*Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria*) and 6 classes (*Actinobacteria, Sphingobacteria, Bacilli*, *α-proteobacteria*, *β-proteobacteria* and *γ-proteobacteria*) have been reported as the taxonomic ranks of cellulolytic bacteria[109], lignin-degrading bacteria[115] and lignocellulolytic bacteria[108, 116]. Woo et al.[108] investigated the diversity of lignocellulolytic Puerto Rican tropical forest soil bacteria which were screened based on the growth on either cellulose or lignin. However, the genus distributions of isolated bacteria in Woo et al.[108] were significantly different from our results. In this work, the major genus of *Actinobacteria* was *Streptomyces*, while the genus *Gordonia* consisted of over 90% of the total *Actinobacteria* (15 out of 16 isolates) in a Puerto Rican rain

Table 4.2.1 Information on the sampling locations

Characteristics	Location	Sample code	GPS coordinate
Mountain soil	Chunggye Mountain	MS-1	37° 24’ 39.9” N 127° 2’ 9.5” E
	Kwanak Mountain	MS-2	37° 28’ 7.3” N 126° 58’ 37.1” E
	Jiri Mountain	MS-3	35° 17’ 48.0” N 127° 31’ 26.9” E
		MS-4	35° 18’ 9.0” N 127° 30’ 52.5” E
	Samin Mountain	MS-5	35° 18’ 49.5” N 126° 54’ 19.8” E
Wetland soil	Upo Wetland	WS-1	35° 32’ 51.0” N 128° 24’ 21.2” E
		WS-2	35° 32’ 55.9” N 128° 25’ 4.7” E
Mudflat soil	Gangwha island	FS	37° 35’ 24.41” N 126° 27’ 26.5” E

Table 4.2.2 Taxonomic analysis of 89 bacterial isolates showing a single or multiple lignocellulolytic activities. The number in parenthesis is the number of isolates affiliated to the corresponding taxonomic rank

Phylum	Class	Order	Family	Genus
Actinobacteria [13]	*Actinobacteria* [13]	*Streptomycetales* [13]	*Streptomycetaceae* [13]	*Streptomyces* [10] *Kitasatospora* [2] *Streptacidiphilus* [1]
Bacteroidetes [2]	*Sphingobacteria* [2]	*Sphingobacteriales* [2]	*Chitinophagaceae* [2]	*Chitinophaga* [1] *Filimonas* [1]
Firmicutes [41]	*Bacilli* [41]	*Bacillales* [41]	*Bacillaceae* [41]	*Bacillus* [41]
Proteobacteria [33]	*α-proteobacteria* [5]	*Caulobacterales* [2] *Rhizobiales* [3]	*Caulobacteraceae* [2] *Phyllobacteriaceae* [3]	*Caulobacter* [2] *Mesorhizobium* [2] *Phyllobacterium* [1]
	β-proteobacteria [11]	*Burkholderiales* [11]	*Burkholderiaceae* [10] *Comamonadaceae* [1]	*Burkholderia* [10] *Variovorax* [1]
	γ-proteobacteria [17]	*Enterobacteriales* [8]	*Enterobacteriaceae* [8]	*Enterobacter* [3] *Klebsiella* [3] *Citrobacter* [1] *Serratia* [1]
		Pseudomonadales [4] *Xanthomonadales* [5]	*Pseudomonadaceae* [4] *Xanthomonadaceae* [5]	*Pseudomonas* [4] *Dyella* [2] *Xanthomonas* [1] *Rhodanobacter* [1] *Luteibacter* [1]

forest soil sample[108]. In addition, *Aquitalea* in *Proteobacteria* was reported to account for 15% of total isolates (75 isolates)[108], but in this work, there was no *Aquitalea* isolate. Those distinct genus level distributions might be attributed to experimental condition variances such as microbial inoculum sources (e.g., sample collecting locations) and the combination of lignocellulose component materials in isolating media (either of cellulose or lignin in Woo et al.'s work[108] vs. including multiple components in this work).

4.3. Taxonomic distribution of bacterial strains by sampling locations

To investigate the influences of ecological conditions in sampling sites on taxonomic distribution of lignocellulolytic bacteria, 4 mountains and 2 wet areas were chosen as lignocellulose-rich sites and lignocellulose-rare sites, respectively. The class-level taxonomic distribution and the number of isolates with respect to the sampling points are shown in Figure 4.3.1. The isolates were distributed widely in 4 classes (MS-1, MS-3, WS-1 and WS-2) or 5 classes (MS-2) except for the isolates from MS-4, MS-5 and FS. Notably, 5 isolates from FS were all *Bacilli*. Although the taxonomic distributions of 5 mountain soil samples were different from those of WS and FS, the class *Bacilli* exhibited the highest abundance in mountain soils (MS-1, MS-2) as well as WS-1, WS-2 and FS. The distributions obtained with the 5 mountain soil samples appeared to be site-specific with different profiles. In particular, even the isolates from MS-3 and MS-4 (1.1 km

apart) sampled from Jiri Mountain showed significantly different taxonomic distributions except for the relative abundance of *γ-proteobacter* (50%). In contrast, the isolates from WS-1 and WS-2 (1.1 km apart) had a similar taxonomic diversity comprising 4 identical classes with *Bacilli* as the most dominant class (over 60% of the isolates from the wetland). When the overall bacterial distributions of lignocellulose-rich dry soil samples (mountain soil samples) were compared with those of lignocellulose-rare wet soil samples (WS-1, WS-2 and FS) (Figure 4.3.2), the isolates belonging to *Actinobacteria*, *Bacilli, α-proteobacteria* and *γ-proteobacteria* were found in both the mountain soils and wet soil samples. In contrast, *Sphingobacteria* and *β-proteobacteria* were isolated only from the mountain soil samples. *Bacilli* had a 3-fold higher isolation possibility from the collected wet soils than from the mountain soils, while there was a 2-fold higher chance to isolate *γ-proteobacteria* from mountain soils than from wet soils (Figure 4.3.2).

In the previous reports, isolation and taxonomic analysis of lignocellulose-degrading bacteria have been conducted mainly with environmental microbial samples adapted under lignocellulose-rich conditions[108, 109]. In this work, lignocellulolytic bacteria were isolated from not only lignocellulose-rich mountain soils but also lignocellulose-rare environmental samples such as wetland soils and mudflat soils. This result implies that microbial inoculum sources for the isolation of lignocellulolytic bacteria are not necessary to be from lignocellulose-abundant (and likely lignocellulose-decaying) sites. Furthermore, considering the different taxonomic abundance and distribution with respect to soil characteristics, use of lignocellulose-lack environmental samples might enable to obtain more diverse

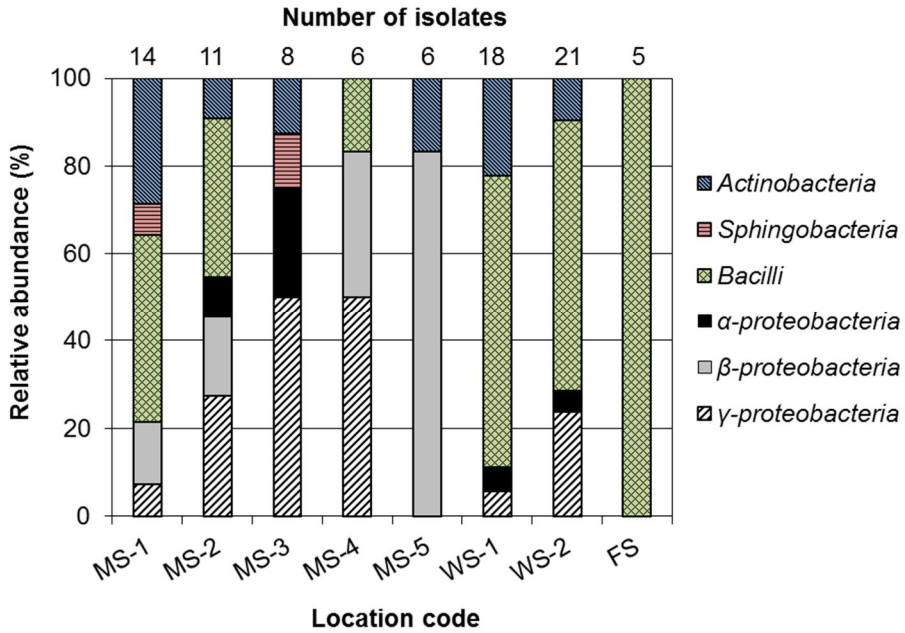


Figure 4.3.1 Relative abundance of bacterial isolates by the sampling locations at the class level.

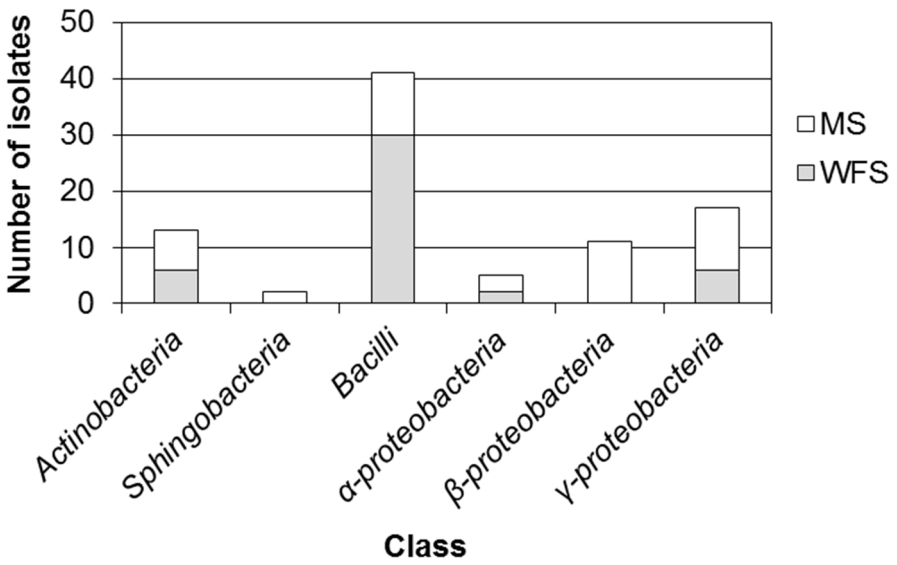


Figure 4.3.2 Taxonomic distribution of isolates between 5 dry mountain soil samples (MS) and 3 wet soil samples (WFS: wetland and mudflat soil samples).

lignocellulolytic bacteria than those previously identified from lignocellulose-rich sites.

4.4. Taxonomic distribution of bacterial strains by carbon sources in isolation media

Next, the microbial distribution of the 89 isolates was analyzed in terms of the carbon sources used in the agar plates: CMC, CRY, CX and CXL. The number of isolates from the CMC, CRY, CX and CXL media was 40, 23, 12 and 14, respectively. As shown in Figure 4.4.1, taxonomic distributions of the isolates from the CMC and CRY media were not much different. The most dominant bacterial class in the cellulose-only media (CMC and CRY media: hereafter CEL) was *Bacilli* (40 isolates), which accounted for over 97% of the total *Bacilli* isolates (41 isolates). However, the class-level distribution of the isolates derived from the CX and CXL media was much different from that of the CEL media indicating a significant effect of the carbon source combinations on the taxonomic distribution. In the case of the CX medium, *Bacilli* isolate was not detected, and *γ-proteobacteria* was the most dominant class (6 isolates; 50% of the CX isolates) followed by *β-proteobacteria* (5 isolates, 42%). Regarding the isolates from the CXL medium, all 6 classes were detected, and *γ-proteobacteria* (6 isolates; 43% of the CXL isolates) was the main class. Because 97% of *Bacillus* (40 out of 41 isolates) and 77% of *Actinobacteria* (10 out of 13 isolates) were isolated from CMC and CRY media (Figure 4.4.1), the CEL media were likely advantageous for isolating *Bacilli* and *Actinobacteria* over the CX or CXL medium. In contrast, with

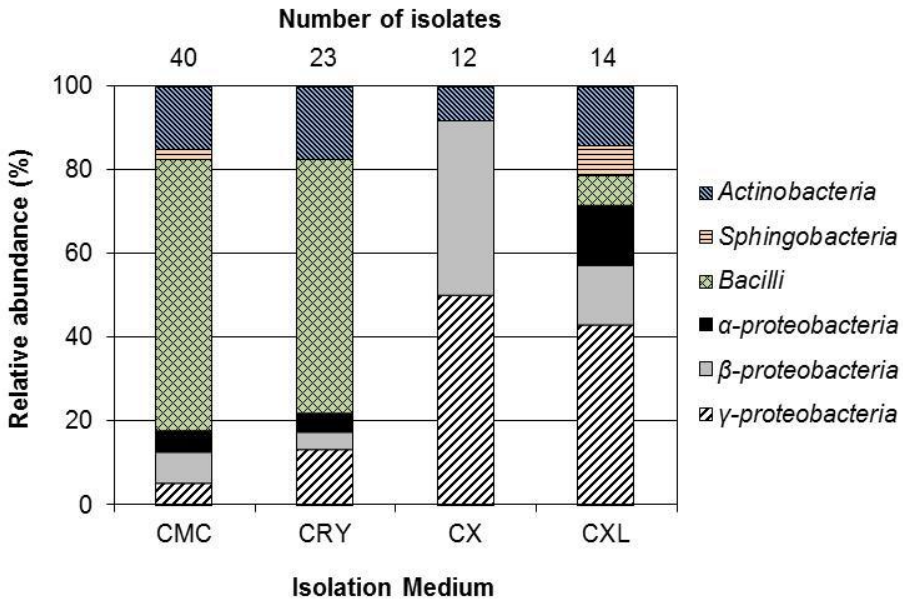


Figure 4.4.1 Relative abundance of bacterial isolates by the combination of lignocellulose components (total concentration of 5 g/L) in the isolation media. CMC, carboxymethyl cellulose; CRY, microcrystalline cellulose; CX, CMC+xylan; CXL, CMC+xylan+alkali lignin

the multiple lignocellulose components (e.g., CX and CXL media), the possibility of isolating *β-proteobacteria* (7 out of 11 isolates) and *γ-proteobacteria* (12 out of 17 isolates) might be higher than with the CEL media.

Isolation of lignocellulolytic bacteria has been previously attempted using agar plates containing a single component of lignocellulose (cellulose, xylan and lignin)[108, 109, 117] . In this study, the mixed lignocellulose components (CX and CXL) as well as cellulose-only media were used as carbon sources in the agar plates for the isolation of lignocellulolytic bacteria. Notably, as shown in Figure 4.4.1, the components of carbon sources in agar plates appeared to greatly affect the taxonomic distributions of lignocellulolytic bacteria, suggesting that the use of mixed lignocellulose components could effective to get lignocellulolytic bacteria rarely discovered with cellulose-only media.

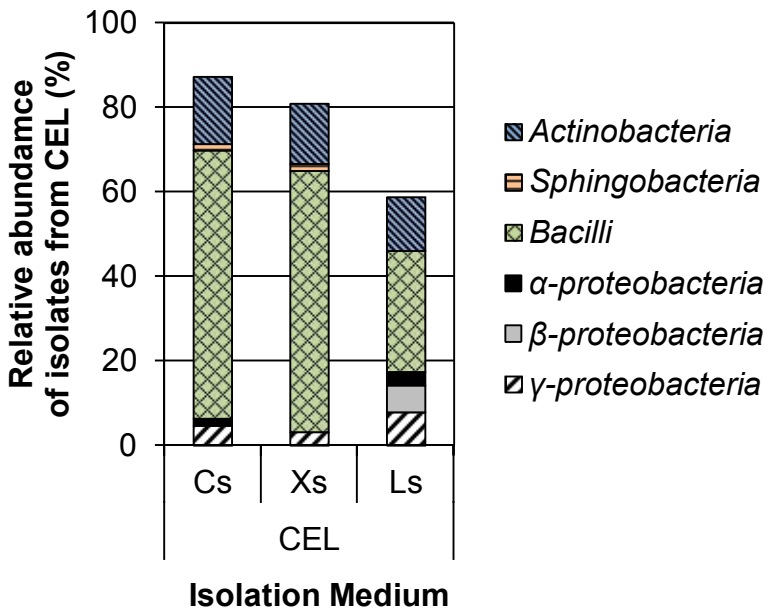
4.5. Screening lignocellulolytic activity of isolates and distribution of activities by the isolation media

Because 89 isolates were able to grow on CMC, CRY, CX and CXL agar media, the cellulolytic (Cs), xylanolytic (Xs) and lignolytic (Ls) activities of the isolates on solid agar plates were assayed. The Cs and Xs were evaluated using Congo red method by detecting halo zones around the colonies on CMC and xylan agar plates. The Ls was estimated by Azure B decolorization and lignin utilization by growth on alkali lignin agar plate as the sole carbon source compared with the growth on agar plate with no added carbon source.

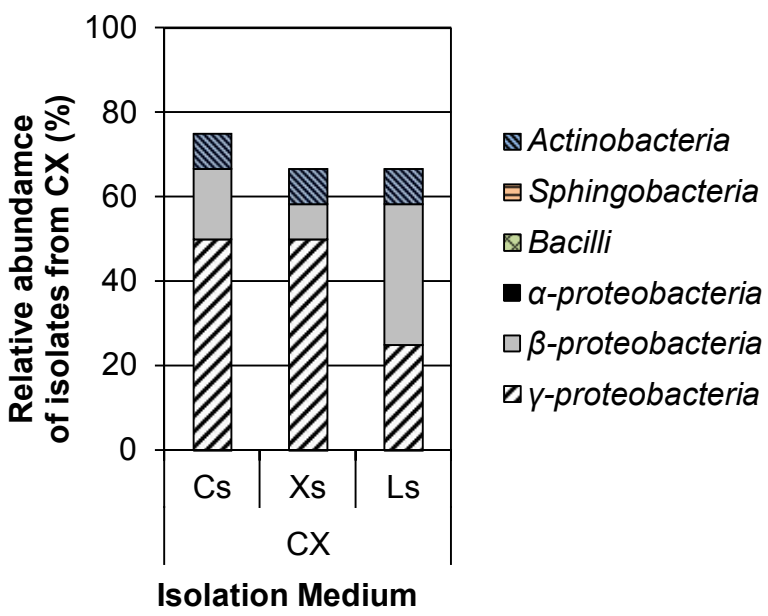
The distributions of the isolates showing Cs, Xs and Ls with respect to the CEL, CX and CXL media are shown in Figure 4.5.1. The sum of the percentage in each medium was over 100% because some isolates exhibited multiple lignocellulolytic activities. As expected, the CEL agar plates were favorable for isolating Cs-exhibiting bacteria, which accounted for over 87% of the CEL isolates. Interestingly, over 80% and 58% of the isolates from the CEL media had Xs and Ls even though there was no xylan and lignin. All *Bacilli* isolates from the CEL media revealed Cs, and up to 50% of *Bacilli* isolates showed Ls. Similarly, Xu and Yang[57] have also found that *S. griseorubens* C-5 isolated using cellulose-based isolation medium exhibited not only cellulase but also xylanase and lignolytic activities such as laccase and peroxidase activities. Unexpectedly, *β-proteobacteria* grown on CEL media exhibited Ls, but did not show Cs probably because of unspecific growth using other nutrients in the agar plates rather than using cellulose. In the case of the CX medium, 75% and 67% of the CX isolates were found to have Cs and Xs, respectively. The portion of Ls-exhibiting isolates was also high (67%), but the taxonomic distribution of isolates with Ls was different from that with Xs: the relative abundance of *β-proteobacteria* significantly increased by 400% while *γ-proteobacteria* population decreased by 50%. Considering that all *β-proteobacteria* bacteria were isolated from the mountain soil samples (Figure 4.3.2) and the relative abundance of *β-proteobacteria* with Ls was higher than those with Cs, the mountain samples were likely more effective for isolating Ls-possessing *β-proteobacteria.*

Regarding the isolates from the CXL medium, a large portion of the isolates (71%) had Ls, whereas only 36% exhibited Cs and Xs in spite of the cellulose and

(a)


Relative abundamce of isolates from CEL (%)
100
80
60
40
20
0
Cs
Xs
Ls
CEL
Isolation Medium
Actinobacteria
Sphingobacteria
Bacilli
α-proteobacteria
β-proteobacteria
γ-proteobacteria

(b)


Relative abundamce of isolates from CX (%)
100
80
60
40
20
0
Cs
Xs
Ls
CX
Isolation Medium
Actinobacteria
Sphingobacteria
Bacilli
α-proteobacteria
β-proteobacteria
γ-proteobacteria

(c)

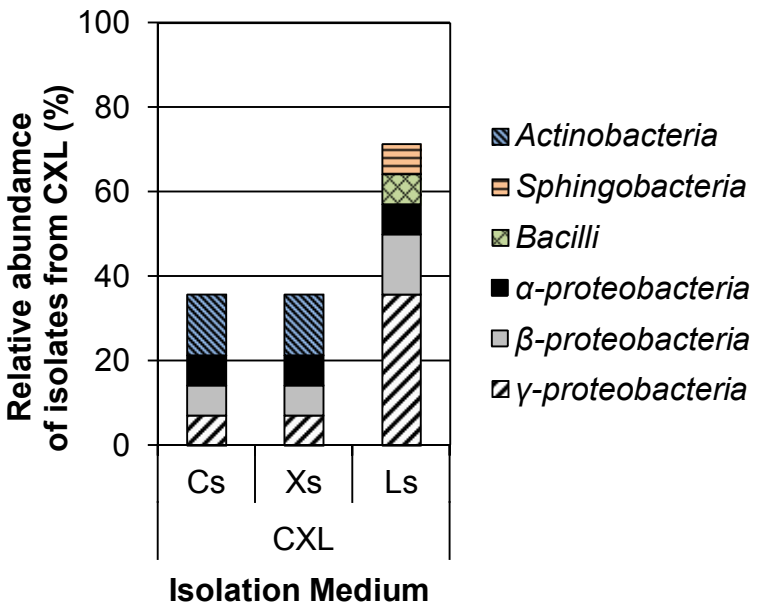


Figure 4.5.1 Distribution of cellulolytic, xylanolytic and lignolytic activities of bacteria isolated from (a) cellulose-only medium (CEL), (b) cellulose-xylan medium (CX) and (c) cellulose-xylan-lignin medium (CXL). Cs, cellulolytic activity; Xs, xylanolytic activity; Ls, lignolytic activity

xylan in the CXL medium. A possible reason for this result could be a toxic effect of lignin on the bacteria. Because lignin is known to be toxic to some bacteria[118, 119], the prerequisite for bacteria to survive on CXL medium is likely a tolerance to lignin rather than having Cs and Xs. Therefore, lignin-absent media might be effective for isolating a wide range of cellulose-degrading bacteria.

The percent compositions of a single activity as well as the multiple lignocellulolytic activities of the isolates from different media are presented as Venn diagrams (Figure 4.5.2 a, b and c). A Venn diagram with all the isolates is shown in Figure 4.5.2d. Regardless of the isolation media, most of the isolates exhibiting Cs were found to have other lignocellulolytic activity such as Xs and Ls. In particular, among Cs-revealing isolates from the CEL media (55 isolates; 87% of CEL media isolates) (Figure 4.5.2a), 54 isolates exhibited multiple activities: 51 isolates (81% of CEL media isolates) with both Cs and Xs (hereafter CsXs); 29 isolates (46%) with both Cs and Ls (hereafter CsLs); and 26 isolates (41%) with Cs, Xs and Ls (hereafter CsXsLS). Only 1% (1 isolate) of Cs-revealing CEL media isolates showed Cs only without any other activities (Figure 4.5.2a). In the case of the Xs, there was no isolate having Xs only and 'XsLs but not Cs' (hereafter XsLs_Cs) (refer to 0% in Figure 4.5.2d). Notably, 100% of the isolates with Xs exhibited Cs as well in all media (i.e., a set of Xs equal to that of CsXs). This result is similar to Ventorino et al.'s report[109] in which xylanase activity was commonly observed along with cellulase activity in lignocellulose-degrading bacteria isolated from wood chips. Additionally, Woo and co-workers screened CMCase and xylanase active bacterial isolates using Congo red agar assay and found that 70% of isolates exhibited both the CMCase and xylanase activities[108]. Those results

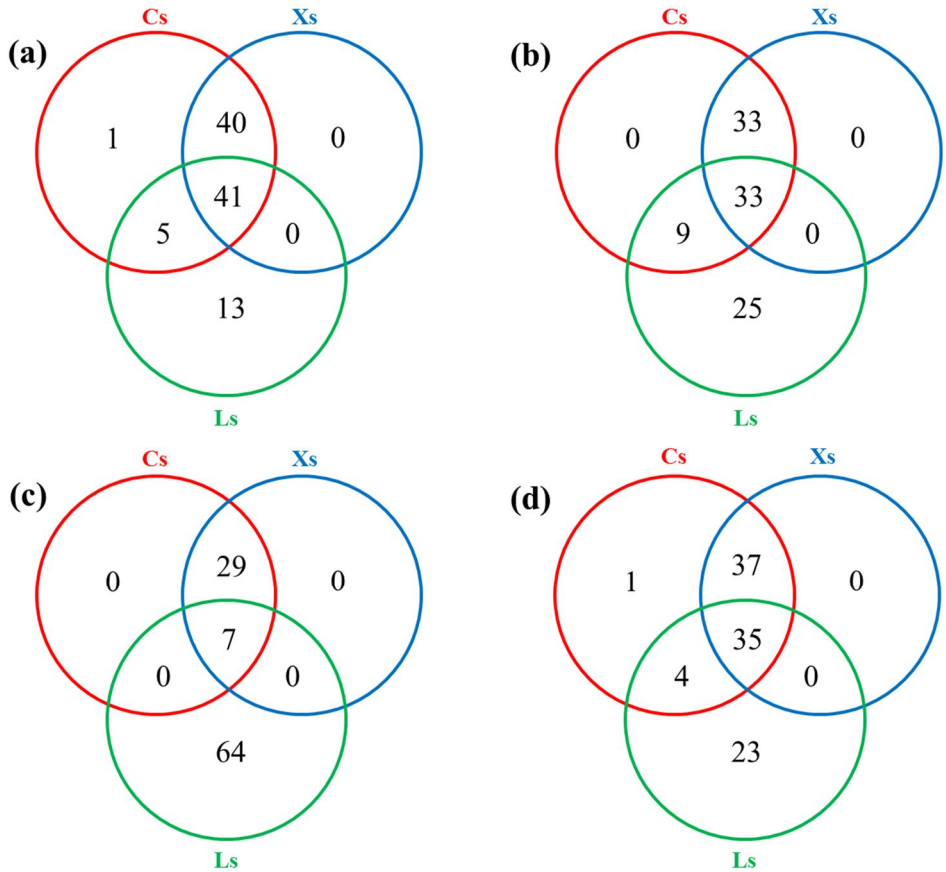


Figure 4.5.2 Venn diagram presenting distribution (%) of bacteria exhibiting single activity and multiple lignocellulolytic activities isolated from (a) cellulose-only medium (CEL, 63 isolates), (b) cellulose-xylan medium (CX, 12 isolates), (c) cellulose-xylan-lignin medium (CXL, 14 isolates) and (d) all the isolation media (CEL, CX and CXL; 89 isolates). Cs, cellulolytic activity; Xs, xylanolytic activity; Ls, lignolytic activity.

imply that xylanolytic bacteria have great potential in degrading cellulose as well in nature. Regarding the isolates with Ls, a large percentage of the Ls-exhibiting isolates from the CEL and CX media showed multiple lignocellulolytic activities such as CsLs and CsXsLs (Figure 4.5.2a and b). However, with the CXL medium, only one-tenth of the Ls-exhibiting isolates (71% of the CXL medium isolates) showed multiple activities of CsXsLs (7% in Figure 4.5.2c). Interestingly, the isolates with Ls only were found even from lignin-absent media such as the CEL (13%, Figure 4.5.2a) and CX (25%, Figure 4.5.2b) media. Even so, considering the much higher percentage of isolates with Ls only on CXL (64%, Figure 4.5.2c), the addition of alkali lignin to the medium seemed advantageous to screen for bacteria with a single activity of Ls. Meanwhile, the portion of isolates with CsXsLs on the CXL medium (7%) was the lowest compared to CsXsLs portions from other media (41% and 33% in CEL and CX media, respectively), which suggests that the use of lignin-containing medium would not be effective in isolating bacteria with CsXsLs.

4.6. Taxonomic distribution of isolates revealing abundantly detected lignocellulolytic activities

Overall, as shown in Figure 4.5.2d, the abundantly observed lignocellulolytic activities were ‘CsXs but not Ls’ (hereafter CsXs_Ls; 37%), followed by CsXsLs (35%) and Ls only (23%). To investigate the relationship between those three major lignocellulolytic activities of isolates and bacterial taxonomic distribution, the most abundant and the second most abundant taxonomic levels were presented

in Table 4.6.1. *Bacillus* was the most dominant genus with CsXsLs and CsXs_Ls, followed by *Streptomyces*. The relative abundances of the sum of the two genera, *Bacillus* and *Streptomyces*, were up to 74% and 76% among the isolates with CsXsLs and CsXs_Ls, respectively. The ratio of *Bacillus* to *Streptomyces* with CsXsLs was 2.8:1, whereas it was 7.3:1 in the bacterial set with CsXs_Ls. The genus *Streptomyces* was relatively abundant in the bacterial set with CsXsLs and CsXs, but no *Streptomyces* was detected among the isolates with Ls only. *Bacillus* has been known to have great potential for using lignocellulose[113, 116, 120]. Some *Bacillus* strains such as *B. pumilus* strain C6 and *B. atrophaeus* strain B7 can degrade fragments of kraft lignin and lignin model compounds[112]; and *B. subtilis* KCTC2023 has a dye-decolorizing peroxidase capable of oxidizing high redox potential substrate (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS], veratryl alcohol [VA] and veratryl glycerol-b-guaiacyl ether [VGE])[121]. Additionally, *Streptomyces* has been well known to degrade lignocellulose[57, 122] and lignin[57, 63, 123] , which is in agreement with the results of this study (Table 4.6.1).

The major genera with Ls only were *Burkholderia* and *Pseudomonas* (Table 4.6.1). These two genera have been known to be closely related in terms of the metabolism of utilizing aromatic compounds[124] which are the common components of lignin[125]. Recently, several *Burkholderia* and *Pseudomonas* have been isolated and characterized to have a lignolytic activity[114, 126, 127]. In class level, *β-proteobacteria* was the most abundant group with Ls only. This result is in agreement with the result in Figure 4.5.1: the relative abundance of *β-proteobacteria* with Ls in all the isolation media was higher than those with Cs and

Table 4.6.1 Distribution of bacterial isolates by lignocellulolytic activities at the genus level

Lignocellulolytic activity			most abundant genus		2nd most abundant genus	
Cellulolytic (Cs)	Xylanolytic (Xs)	Lignolytic (Ls)	Genus (Class)	Relative abundance (%)	Genus (Class)	Relative abundance (%)
O	O	O	*Bacillus* (*Bacilli*)	55	*Streptomyces* (*Actinobacteria*)	19
O	O	X	*Bacillus* (*Bacilli*)	67	*Streptomyces* (*Actinobacteria*)	9
X	X	O	*Burkholderia* (*β-proteobacteria*)	40	*Pseudomonas* (*γ-proteobacteria*)	15

Xs in Figure 4.5.1. Given that *β-proteobacteria* isolates were found only in mountain soil samples, lignocellulose-rich mountain samples rather than the other lignocellulose-rare samples appeared to be advantageous to isolate *β-proteobacteria* with Ls only.

4.7. Conclusion

Lignocellulolytic bacteria were isolated from mountain, wetland and mudflat soil samples using different combinations of lignocellulose components. Also, the influence of isolation methodology was investigated on lignocellulolytic activities and bacterial taxonomic distributions. Lignocellulolytic bacteria were isolated from not only lignocellulose-rich mountain soils but also lignocellulose-lack wet soil samples. Cellulose-only media were effective to isolate bacteria with Cs as well as multienzymatic activities in spite of the absence of xylan and lignin. Lignin-containing medium was effective to isolate bacteria with Ls only, but not favorable to isolate CsXsLs. *Bacillus* and *Streptomyces* accounted for over 70 % of CsXs-revealing bacteria and the most abundant genus with Ls only was *Burkholderia* (belonging to the class *β-proteobacteria*) isolated only from mountain soil samples in this study. Notably, high CsXsLs activity-possessing bacteria were isolated from lignocellulose-rich mountain soils as well as lignocellulose-lack wet soil samples. Overall, the sampling locations and the isolation medium were important experimental factors to efficiently isolate bacteria with specific lignocellulolytic activities.

Chapter 5.

Selection of high lignocellulolytic potential bacterium *Bacillus* sp. 275 isolated from mud flat

5.1. Introduction

The development of effective selection methods for bacterial isolates is important for utilizing lignocellulosic biomass. In general, the decolorization of various synthetic dyes (e.g. model compounds) is useful for detection of lignin degradation. In addition, the colorimetric assay called CRACC (Congo Red Analysis of Cellulose Concentration) was developed for measurement of cellulose degradation by Haft and co-authors[128]. These colorimetric methods are rapid, sensitive and simple, in general. However, the degradation patterns or degree between theses model compounds and the real samples such as lignocellulosic biomass, crystal cellulose, filter paper and alkali lignin are different.

In this chapter, lignocellulose-degrading bacteria, *Bacillus* sp. 275 was successfully selected using combination of colorimetric methods using Congo red and Azure B, and direct growth in cellulose, xylan and lignin media. Moreover, lignocellulolytic enzyme activities such as cellulase, xylanase, laccase and peroxidase were measured.

5.2. Identification of selected isolates exhibiting relatively high cellulolytic, xylanolytic and lignolytic activities in solid media

Among the 89 isolates, 31 isolates showed CsXsLs. To investigate whether the specific genera, isolation medium, and sampling points were related to the high

level of CsXsLs, 10 bacterial strains revealing relatively high CsXsLs were selected. The genera of the selected strains were *Bacillus* (4 isolates), *Streptomyces* (3 isolates), *Kitasatospora, Klebsiella* and *Burkholderia* (1 isolate each) (Table 5.2.1). *Bacillus* and *Streptomyces* accounted for 70% of the selected isolates, which was similar to the relative abundance of these two genera in the set of CsXsLs (Table 4.6.1). The ratio of the isolates with the high CsXsLs from the CEL, CX, and CXL media was 7:2:1 (Table 5.2.1). Regarding sampling locations, 5 isolates were obtained from the mountain samples (MS-1, MS-2 and MS-5), 2 isolates from the wetland (WS-1 and WS-2), and 3 isolates from the mudflat soil (FS). This result implies that bacteria with relatively high level of CsXsLs can be isolated from not only lignocellulose-rich sites (e.g., mountain) but also lignocellulose-rare environmental samples containing a diverse microbial community (e.g., wetland or mudflat soil).

While selected strains showed high lignocellulolytic activities on solid media, there were differences in the mount of extracellular protein. Strain 275 has the highest concentration of extracellular protein (74.48 mg/L), followed by strain 272 (64.87 mg/L) and strain 313 (51.15 mg/L). In terms of lignin degrading ability, half of bacterial strains have lignolytic enzyme activities like laccase or peroxidase. However, the other half of isolates were lacked laccase and peroxidase, though they grew well in lignin agar plate. Despite relative low concentration of extracellular protein, strain 414 exhibited cellulase, xylanase and laccase activities. The strain with the highest laccase and peroxidase activity was stain 414 (7.2 U/g) and 594 (1.7 U/g), respectively. Interestingly, strain 275 showed extraordinary activities of cellulase and xylanase compared with other isolates.

Table 5.2.1 Identification and lignocellulolytic activities of the selected isolates

Bacterial isolates	Class	Identification	Similarity (%)	Cellulase	Xylanase	Ligninase	Isolation medium	Isolation location
123	*Bacilli*	*Bacillus aryabhattai*	99.86	++	++	+++	CMC	MS-1
272	*Bacilli*	*Bacillus aryabhattai*	99.65	++	++	++	CMC	FS
273	*Bacilli*	*Bacillus megaterium*	99.79	++	++	++	CMC	FS
275	*Bacilli*	*Bacillus siamensis*	99.86	++	++	+++	CMC	FS
414	*Actinobacteria*	*Streptomyces durhamensis*	98.85	+++	++	+++	CMC	WS-1
132	*Actinobacteria*	*Streptomyces olivochromogenes*	99.71	+++	++	++	CRY	MS-1
331	*Actinobacteria*	*Streptomyces indigoferus*	99.08	+++	++	+++	CRY	MS-2
856	*Actinobacteria*	*Kitasatospora griseola*	98.23	+++	+++	++	CX	MS-5
594	*γ-proteobacteria*	*Klebsiella michiganensis*	99.48	++	++	+++	CX	WS-2
815	*β-proteobacteria*	*Burkholderia ubonensis*	98.78	+++	+++	++	CXL	MS-5

++: medium activity, +++: strong activity

Table 5.2.2 Lignocellulolytic activities of supernatant of the selected isolates grown in liquid medium

Bacterial isolates	Protein conc. (mg/L)	Cellulase (U/g)	Xylanase (U/g)	Laccase (U/g)	Peroxidase (U/g)
123	28.00	474.2	52.7	N.D.	N.D.
272	64.87	196.1	21.8	3.3	1.1
273	23.25	551.1	61.2	N.D.	N.D.
275	74.48	1245.8	138.4	1.3	0.5
414	28.32	468.5	52.1	7.2	N.D.
132	20.33	607.7	67.5	N.D.	N.D.
331	51.15	245.0	27.2	5.6	0.9
856	36.39	346.2	38.5	N.D.	N.D.
594	48.36	245.5	27.3	3.4	1.7
815	35.49	334.5	37.2	N.D.	N.D.

N.D.: Not Detected.

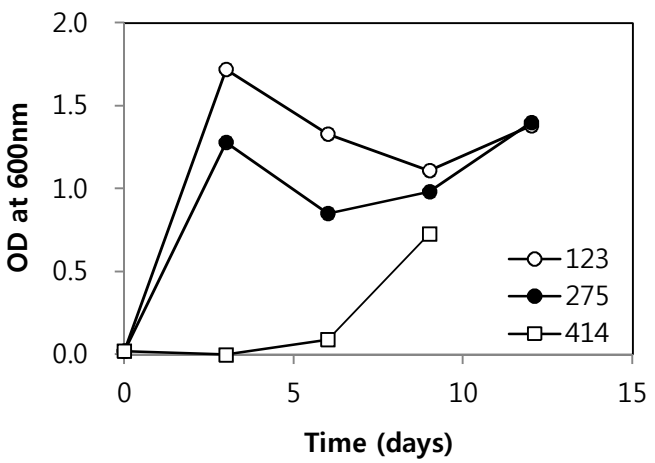
5.3. Bacterial growth in liquid lignin medium and mixed carbon medium consist of cellulose, xylan and lignin

As mentioned in section 5.2., ten bacterial isolates revealed relatively high lignocellulolytic activities on solid media and in liquid LB medium. Next, the microbial growth was tested in culture medium based on lignocellulosic carbon sources like cellulose, xylan and lignin. Among ten isolates, only three isolates (strain 123, 275 and 414) were grown in mixed carbon medium consisting of cellulose, xylan and lignin (CXL medium). Moreover, these three isolates were grown in alkali lignin medium as a sole carbon sources. The other seven isolates did not grow in these two kinds of carbon sources in culture medium.

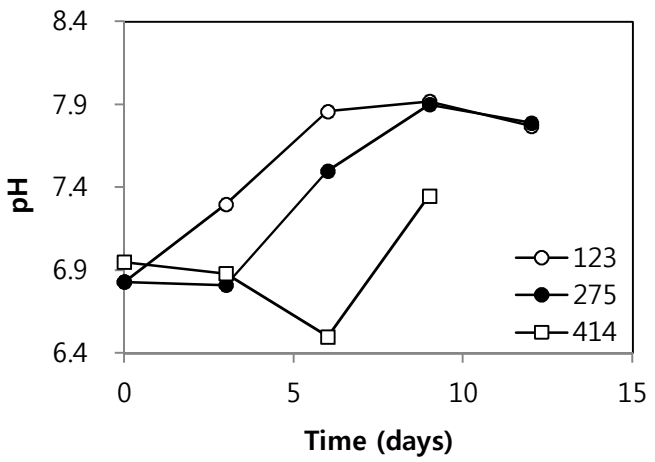
The growth of strain 123 and 275 was faster and higher than strain 414 in CXL medium. In the case of strain 414, the cell aggregation was observed after 9 days incubation. Regarding to growth in CXL medium, strain 123 was the best strain compared to strain 275 and 414. The maximum cell growth of all three strains was lower at alkali lignin medium compared to CXL medium. The growth of strain 275 was the highest among three bacterial isolates. However, the growth of strain 123 which was the highest in CXL medium was the lowest in alkali lignin medium. This results imply that strain 123 utilizes cellulose and xylan more efficiently than lignin.

5.4. Analysis of lignocellulolytic degrading enzymes of selected isolates

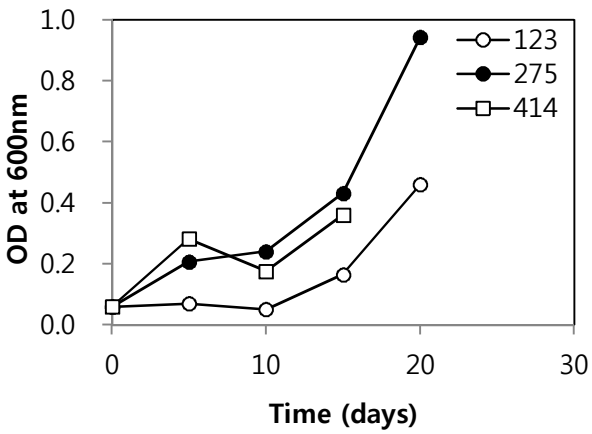
(a)


2.0
1.5
1.0
0.5
0.0
OD at 600nm
0
5
10
15
Time (days)
123
275
414

(b)


8.4
7.9
7.4
6.9
6.4
pH
0
5
10
15
Time (days)
123
275
414

(c)



(d)

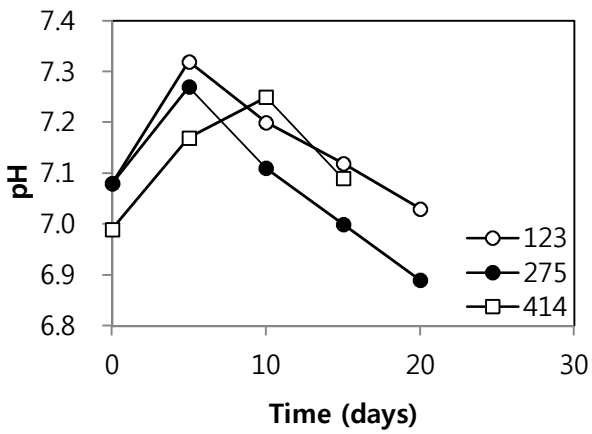


Figure 5.3.1 Time course of cell growth and pH in CXL (a, b) and alkali lignin medium (c, d).

Because of the growth of strain 123, 275 and 414 in CXL and lignin media, the lignocellulolytic enzyme activities were analyzed with respect to culture media with different carbon sources. The enzyme activities of cellulase, xylanase and peroxidase for degradation of cellulose, hemicellulose (especially xylan) and lignin were measured.

As shown in Table 5.4.1, all three strains showed xylanase activity. However, none of them showed cellulase activity. These results imply that these bacterial isolates have xylanolytic activity but cellulolytic activity is relatively low. Because all three isolates showed cellulase activity in the supernatant of LB growth medium, cell growth or extracellular proteins were not enough to detect cellulase activity in CMC-based medium. Regarding to peroxidase activity, strain 275 showed the distinguished feature than strain 123 and 414. Although the supernatant of strain 414 cultured in CXL medium showed peroxidase activity, stain 275 showed peroxidase activity in supernatant of xylan, CXL and lignin media. This result was correlated with the high cell growth of 275 in lignin medium (Figure 5.3.1).

5.5. Oxidation of veratryl alcohol in selected isolates and *Bacillus* species

Veratryl alcohol (3,4-dimethoxybenzyl alcohol) is one of the main substrate of lignin-degrading enzyme, especially, lignin peroxidase. Oxidation of veratryl alcohol to veratryl aldehyde (3,4-dimethoxybenzaldehyde) with hydrogen peroxide (H_2O_2) using enzyme of interest is considered as a promising results for lignin

Table 5.4.1 Lignocellulolytic activities of thee selected isolates grown in different carbon sources

Activity	Growth medium	Bacterial isolates		
		123	275	414
Peroxidase	Xylan	X	O	X
	CXL	X	O	O
	Lignin	X	O	X
Cellulase	CMC	X	X	X
Xylanase	Xylan	O	O	O

O = detected, X = not detected

degradation by that enzyme.

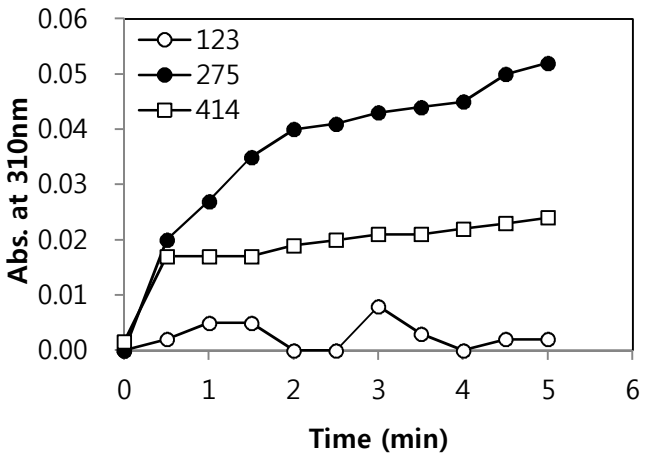
Among the three selected isolates, only strain 275 showed an ability of veratryl alcohol oxidation. Stain 123 and 414 did not show the increase of absorbance at 310nm, which means the increase of veratryl aldehyde concentration. As the results, strain 275 was finally selected candidate for lignocellulolytic bacterium.

Because of strain 275 identified as *Bacillus* species (Table 5.2.1), veratryl alcohol oxidation test was conducted using cell-free supernatant of other two *Bacillus* species (*B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580). As shown in Figure 5.5.1, strain 275 exhibited high veratryl alcohol oxidation activity, but small increase in *B. subtilis* ATCC6051, and no increase in *B. licheniformis* ATCC14580.

5.6. Conclusion

In this chapter, candidate bacterial isolates were selected for degradation of lignocellulose among 89 isolates from various environmental sites as mentioned in chapter 4. The first step is a selection of isolates showed relatively high cellulolytic, xylanolytic and lignolytic activities on solid media. As a result, ten isolates (four *Bacillus*, three *Streptomyces*, one *Kitasatospora, Klebsiella* and *Burkholderia*) were selected in the first round. All ten isolates showed cellulase and xylanase activities in the LB medium supernatant. However, some isolates did not show laccase or peroxidase. In the case of CXL or alkali lignin media, only three isolates (123, 275 and 414) can be grown.

(a)



(b)

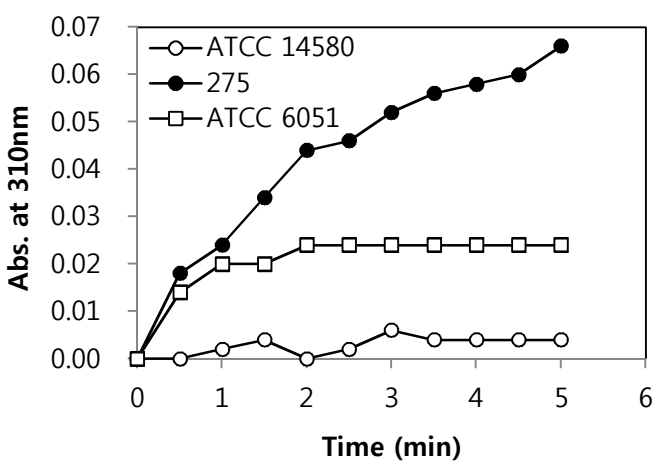


Figure 5.5.1 Veratryl alcohol oxidation by three selected isolates (a) and *Bacillus* species (b).

Based on the results of growth in CXL and alkali lignin media, and lignocellulolytic enzyme activities including cellulase, xylanase and peroxidases, and oxidation of veratryl alcohol, stain 275 was finally chosen for the further detailed investigation.

Chapter 6.

Genomic analysis of *Bacillus* sp. 275

6.1. Introduction

Bacillus is a genus of rod-shaped, Gram-positive, aerobic or facultative anaerobic bacteria consisting of a very diverse group of over 300 species (List of Prokaryotic names with Standing in Nomenclature, http://www.bacterio.net/bacillus.html). Several *Bacillus* strains have been reported to degrade lignin or lignin model compounds[129-131]. Moreover, *Bacillus* sp. 55S5 isolated from peat exhibited not only the capability of lignin modification but also cellulase and xylanase activities[132]. Similarly, *Bacillus* sp. R2 isolated from the Red Sea also had cellulase, xylanase, pectinase and peroxidase activities[133]. Those results imply that *Bacillus* species have potential in degrading lignocellulose components including not only lignin but also cellulose and hemicellulose.

In this study, the complete genome of *Bacillus* sp. 275, a potential lignocellulose-degrading bacterium, was sequenced and analyzed its genes related to the lignocellulolytic activities.

6.2. Genome summary of *Bacillus* sp. 275

The complete genome of *Bacillus* sp. 275 consisted of one 4,045,581 bp chromosome and one 6,389 bp plasmid with 3,832 protein cording sequences (CDS), 86 tRNA genes, 27 rRNA genes and an average G+C content of 46.32% (Table 6.2.1 and Figure 6.2.1). The NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) was used to annotate the genes of *Bacillus* sp. 275.

Table 6.2.1 Genome features of *Bacillus* sp. 275 strain

Features	Chromosome	Plasmid
Genome size (bp)	4,045,581	6,389
G + C content (%)	46.33	43.25
Total number of genes	4,017	6
Protein-coding genes (CDS)	3,827	5
rRNA genes	27	0
tRNA genes	86	0

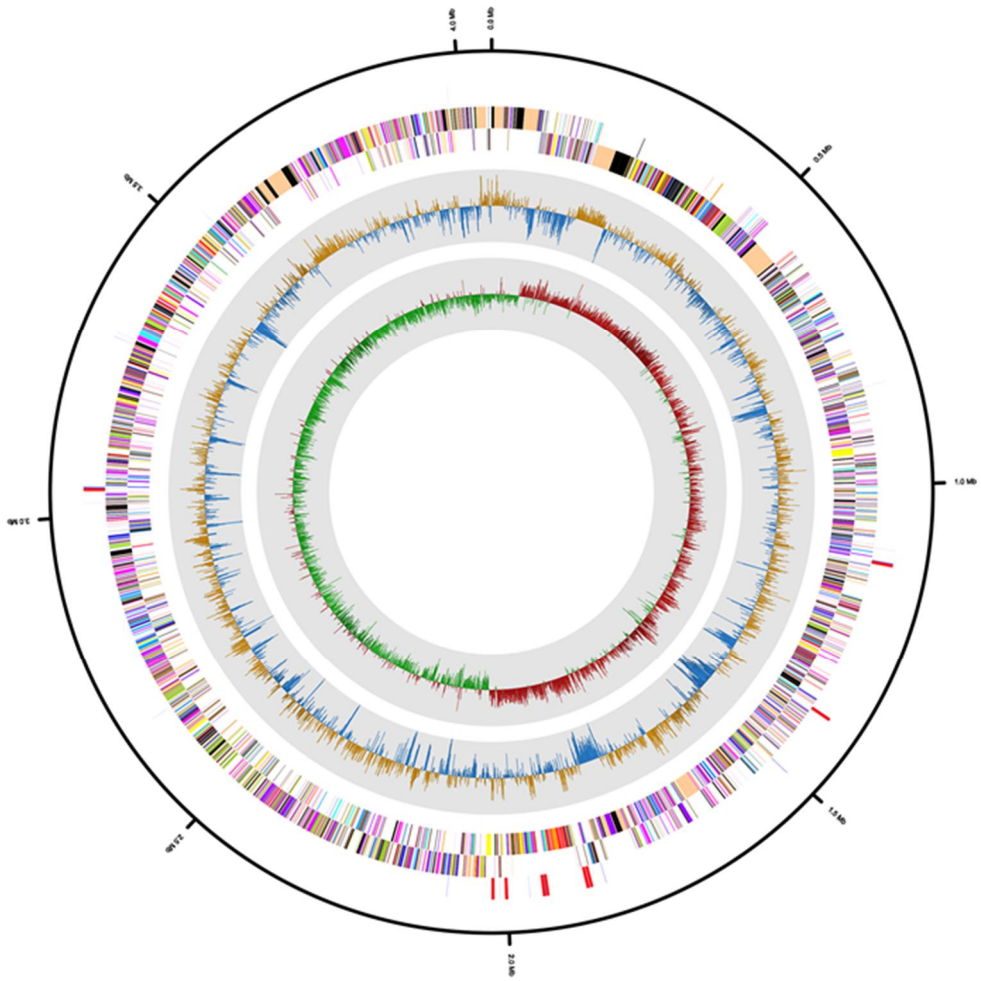


Figure 6.2.1 Circular genome map of *Bacillus* sp. 275. The circular genome map consists of 5 circles. From the outer circle inward, each circle displays information about the genome of rRNA/tRNA, Reverse CDS, Forward CDS, G + C Ratio and GC Skew.

6.3. Identification of *Bacillus* sp. 275

Strain 275 was initially classified as *B. siamensis* based on the most hit taxon strain with the 16S rRNA sequence data in the Eztaxon server (http://www.ezbiocloud.net)[134] compared to *B. siamensis* KCTC 13613^T with a pairwise similarity of 99.93% (e.g., 1471 bp matching out of 1472 bp). However, the number of mismatching nucleotide between the strain 275 and *B. velezensis* CR-502^T was also only one bp (e. g., 1402 bp matching out of 1403 bp) as same as the *B. siamensis* KCTC 13613^T. Meanwhile, the average nucleotide identity (ANI) values of the strain 275 compared to *B. velezensis* FZB42 were found to be over 98%. *B. velezensis* FZB42 had formerly classified as *B. amyloliquefaciens* subsp. *plantarum* FZB42^T and it had been the closest type strain to *Bacillus* sp. 275 based on the 16S rRNA sequences until *B. amyloliquefaciens* subsp. *plantarum* FZB42^T was re-classified to *B. velezensis* FZB42[135]. Based on these results that the closest relative to the strain 275 could not be clearly defined, the strain 275 was named as *Bacillus* sp. 275.

A phylogenetic tree of *Bacillus* sp. 275 based on 16S rRNA sequences is shown in Figure 6.3.1. *Bacillus* sp. 275 was closely related to *B. siamensis* KCTC 13613^T and *B. velezensis* CR-502^T as described earlier. Furthermore, the tree showed that *B. amyloliquefaciens* DSM 7^T formed a distinct branch together with *B. siamensis* KCTC 13613^T, *B. velezensis* CR-502^T and *Bacillus* sp. 275.

6.4. Lignocellulose-degrading genes in *Bacillus* sp. 275

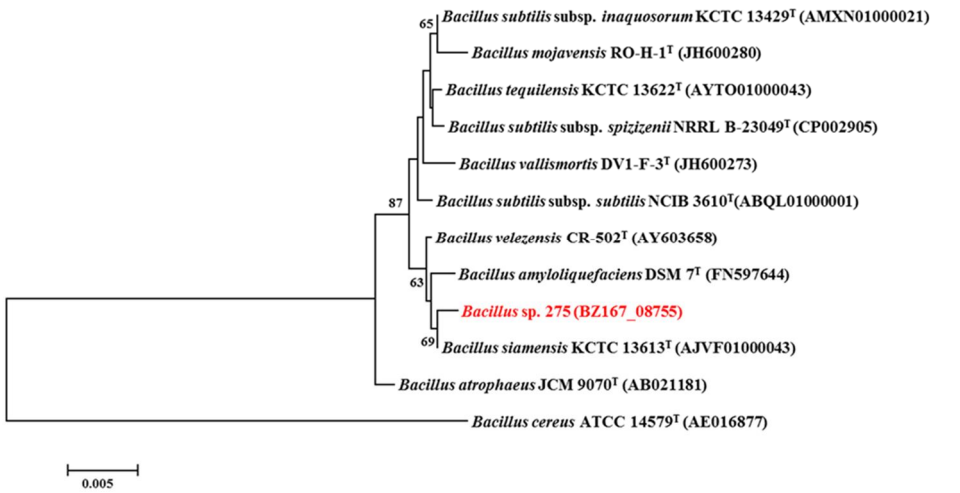


Figure 6.3.1 Phylogenetic tree derived from 16S rRNA gene sequence data of *Bacillus* sp. 275 and its relatives. The tree was generated by the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA7 and the distances were computed using the Maximum Composite Likelihood method. *Bacillus cereus* ATCC 14579 was used as the out group. Only bootstrap values above 60% were shown from 1000 replications. Bar, 0.005 nucleotide substitutions per site.

The genes related to the degradation of lignocellulose were detected in the genome of *Bacillus* sp. 275 (Table 6.5.2). Endoglucanase, β-glucanase, glucohydrolase and glucosidase were found in the complete genome of *Bacillus* sp. 275. Regarding xylan degradation, glycoside hydrolase 43 family protein (1,4-β-xylosidase), arabinoxylan arabinofuranohydrolase, glucuronoxylanase and α-N-arabinofuranoside were found. The genes encoding deferrochelatase (dye decolorizing peroxidase) and laccase involved in lignin degradation were also observed in the genome of *Bacillus* sp. 275. In addition, *Bacillus* sp. 275 has other glycosidases including α-amylase, β-mannosidase, endo-1,4-β-galactanase, 6-phospho-β-galactosidase, arabinan endo-1,5-α-L-arabinosidase and endo-α-(1->5)-L-arabinanase. The existence of these genes implies that strain 275 has the potential for utilizing or degrading starch, mannan, galactoside and arabinan.

6.5. Comparative analysis of genes related to lignocellulose degradation in *Bacillus* species

The complete genomes of *B. siamensis* KCTC 13613^T and *B. velezensis* CR-502^T have not been reported. Therefore, instead of *B. siamensis* KCTC 13613^T and *B. velezensis* CR-502^T, *B. siamensis* SDLI1 (the only *B. siamensis* strain with the complete genome sequences available) and *B. velezensis* FZB42 whose complete genome sequences have been reported were selected for the comparative genome analysis with *Bacillus* sp. 275. Additionally, the complete genome of *B. amyloliquefaciens* DSM 7^T was also compared with that of *Bacillus* sp. 275.

The COG functional categories of the four complete genome sequences are shown in Table 6.5.1. Among the 3,832 CDS of *Bacillus* sp. 275, 3,431 CDS were classified into COG categories[136]. The major categories of *Bacillus* sp. 275 were transcription (K), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), energy production and conversion (C), cell wall/membrane/envelope biogenesis (M) and inorganic ion transport and metabolism (P). Especially, the number of genes in carbohydrate transport and metabolism (G) related to lignocellulose degradation were 241. This value is similar to that of *B. velezensis* FZB42, but higher than those of *B. siamensis* SDLI1 and *B. amyloliquefaciens* DSM 7^T by 9% and 3%, respectively.

Most of the genes related to the degradation of lignocellulose were highly observed in all strains (Table 6.5.2). However, in the genome of *B. amyloliquefaciens* DSM 7^T, endoglucanase, α-amylase and 1,4-β-xylosidase were not found. The distribution of lignocellulose degradation-related genes between *Bacillus* sp. 275 and *B. siamensis* SDLI1 were also somewhat different. In our study, all lignocellulose-degrading genes in *Bacillus* sp. 275 were found in *B. velezensis* FZB42 except 6-phospho-β-glucosidase (BglH). *B. velezensis* has been mainly studied for its relationship with plant and fungi, because *B. velezensis* produces antifungal secondary metabolites[137].

6.6. Conclusion

In conclusion, *Bacillus* sp. 275 has the cellulolytic, xylanolytic and ligninolytic enzyme activities and also has genes encoding lignocellulolytic

Table 6.5.1 COG functional categories of the complete genome sequences of *Bacillus* sp. 275, *B. siamensis* SDLI1, *B. amyloliquefaciens* DSM 7^T and *B. velezensis* FZB42

Code	Functional annotation	Strains (number of genes)			
		275	SDLI1	DSM 7^T	FZB42
J	Translation, ribosomal structure and biogenesis	159	163	161	159
K	Transcription	261	255	271	261
L	Replication, recombination and repair	159	157	155	140
D	Cell cycle control, cell division, chromosome partitioning	35	36	36	36
O	Posttranslational modification, protein turnover, chaperones	100	103	108	102
M	Cell wall/membrane/envelope biogenesis	183	210	206	195
N	Cell motility	47	42	45	42
P	Inorganic ion transport and metabolism	181	179	180	188
T	Signal transduction mechanisms	140	138	136	143
C	Energy production and conversion	184	179	176	180

G	Carbohydrate transport and metabolism	241	222	235	244
E	Amino acid transport and metabolism	284	284	299	293
F	Nucleotide transport and metabolism	77	90	84	80
H	Coenzyme transport and metabolism	102	108	108	111
I	Lipid transport and metabolism	110	110	104	108
Q	Secondary metabolites biosynthesis, transport and catabolism	91	83	67	87
S	Function unknown	1077	1174	1200	1074
Total		3431	3533	3571	3443

Table 6.5.2 Comparison of genes encoding lignocellulose-degrading enzymes in *Bacillus* sp. 275 and other *Bacillus* strains

Bacillus sp. 275		SDLI1	DSM 7^T	FZB42
Predicted function	Accession No.			
Cellulose-related				
endoglucanase	AQP94520	O	X	O
β-glucanase	AQP96424	O	O	O
glucohydrolase	AQP97176	O	O	O
glucohydrolase	WP_077392024	O	O	O
6-phospho-α-glucosidase	AQP95497	O	O	O
6-phospho-β-glucosidase	AQP96452	X	O	O
6-phospho-β-glucosidase	WP_077392079	O	O	O
6-phospho-β-glucosidase	AQP98009	O	O	O
α-glucosidase	AQP97260	X	O	O
aryl-phospho-β-D-glucosidase	AQP95939	X	O	O
α-amylase	AQP95972	O	X	O
Hemicellulose-related				

glycoside hydrolase 43 family protein	AQP94597	O	X	O
arabinoxylan arabinofuranohydrolase	AQP94515	O	O	O
glucuronoxylanase	WP_077391892	O	O	O
arabinan endo-1,5-α-L-arabinosidase	AQP97388	X	O	O
endo-α-(1->5)-L-arabinanase	WP_077392066	O	O	O
α-N-arabinofuranoside	AQP97416	O	O	O
α-N-arabinofuranoside	AQP97396	O	O	O
β-mannosidase	AQP96449	X	O	O
endo-1,4-β-galactanase	AQP95141	O	O	O
6-phospho-β-galactosidase	WP_077391940	O	O	O
Lignin-related				
deferrochelatase	AQP96486	O	O	O
laccase	AQP94816	O	O	O

O = detected (over 90% sequence homology); X = not detected (below 40% sequence homology)

enzymes. The complete genome information of *Bacillus* sp. 275 would enhance a better understanding on the degradation of lignocellulose in the genus *Bacillus*. These results would provide insight into bacterial degradation of all components in lignocellulose and possibly a new use for *Bacillus* sp. 275 in the conversion of lignocellulose into useful chemicals and fuels.

Chapter 7.

Lignocellulose degradation by

Bacillus sp. 275

7.1. Introduction

In previous chapters, potential lignocellulolytic bacteria were isolated and selected from various environmental soil samples. Finally, one isolate was chosen as a promising bacterium for degradation of lignocellulose (e.g., cellulose, xylan and lignin). The genome of isolate has genes encoding cellulase, xylanase, peroxidase and laccase which are related to the lignocellulose degradation.

Based on these results, the degradation ability of lignocellulosic biomass was characterized by isolate named as *Bacillus* sp. 275 from mudflat soil, in this chapter. The cell growth in complex, nutrient-rich medium and defined medium was compared with or without lignin in the growth medium. Regarding the cellulose and xylan, the products of polysaccharide degradation were observed by TLC and HPLC. The growth and enzyme activities were also monitored in xylan-based medium. In addition, the degradation aspect of filter paper by *Bacillus* sp. 275 was studied.

7.2. Cellulolytic, xylanolytic and lignolytic activities by decolorization and growth comparison in solid media

Bacillus sp. 275 was isolated from mudflat soil with a high salt concentration and the carbon source of isolation medium was carboxymethyl cellulose. *Bacillus* sp. 275 showed relatively high lignocellulolytic activities on solid agar plates during screening of lignocellulose-degrading bacteria.

Cellulase activity was measured with the agar plates containing carboxymethyl cellulose and microcrystalline cellulose using Congo red staining to analyze halo zone around the colony. As shown in Figure 7.2.1, *Bacillus* sp. 275 exhibited clear halo zones both carboxymethyl cellulose and microcrystalline cellulose agar plates. These results imply that *Bacillus* sp. 275 could be degraded and utilized the insoluble cellulose (e.g., microcrystalline cellulose) besides easily available soluble cellulose (e.g., carboxymethyl cellulose). The xylanase activity also detected on agar plate with xylan as a sole carbon source using Congo red staining method mentioned above. Regarding the lignin utilization, the colorimetric analysis using Azure B, a lignin analogue and the observation of direct utilization of alkali lignin were measured. The decolorization of Azure B was clearly detected (Figure 7.2.1d) and the growth of *Bacillus* sp. 275 in agar plate using alkali lignin as a sole carbon source was observed (Figure 7.2.1e). On the other hand, *Bacillus* sp. 275 was not able to use agar as a carbon source (Figure 7.2.1f). Therefore, *Bacillus* sp. 275 undoubtedly utilizes lignin as a carbon source by combination of the results with Figure 7.2.1 e and f.

Based on the results on solid agar plates, *Bacillus* sp. 275 exhibited the lignocellulolytic activities such as cellulase, xylanase and ligninase.

7.3. Modification and utilization of alkali lignin during growth in complex and defined liquid media

The effect of the alkali lignin on the growth of *Bacillus* sp. 275 was

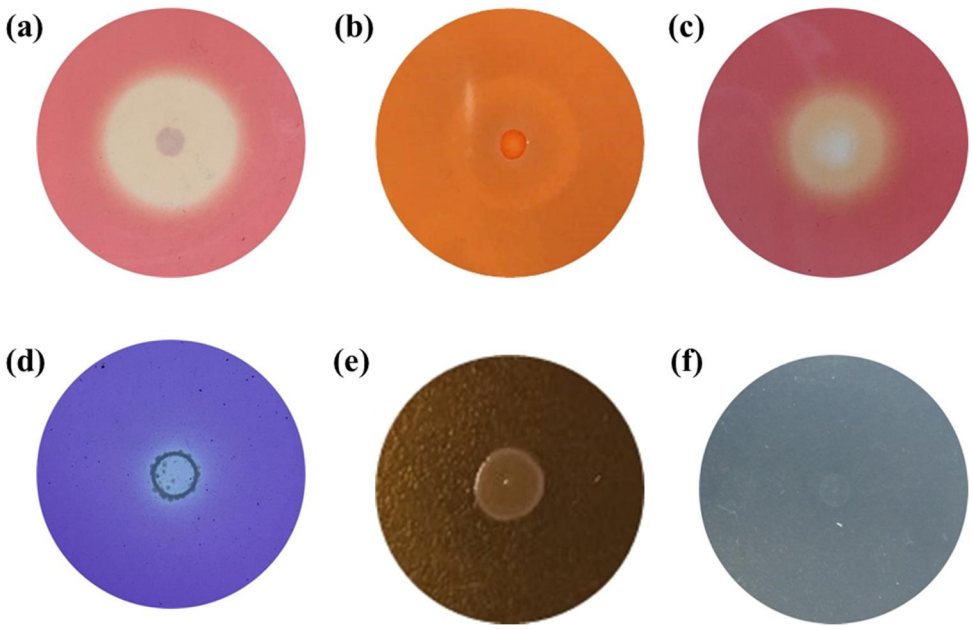
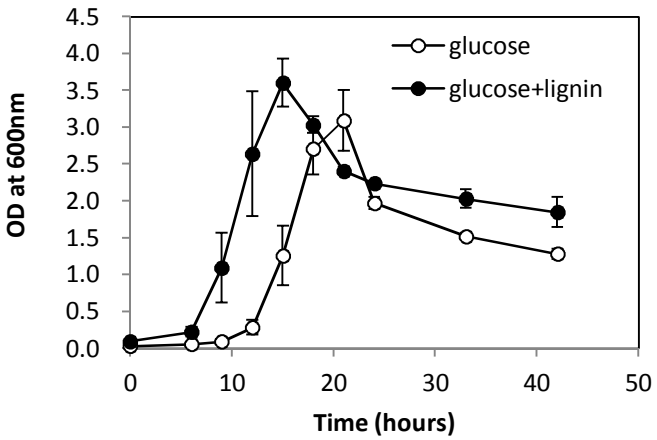


Figure 7.2.1 Lignocellulolytic activities of *Bacillus* sp. 275 on agar plates. Cellulase activity was detected on CMC (a) and crystalline cellulose (b) agar plates and xylanases activity on xylan agar plate (c) using Congo red staining method. Lignin utilizing ability was observed by decolorization on Azure B (d) and growth on lignin agar plate (e) compared to only-agar plate without any carbon sources (f).

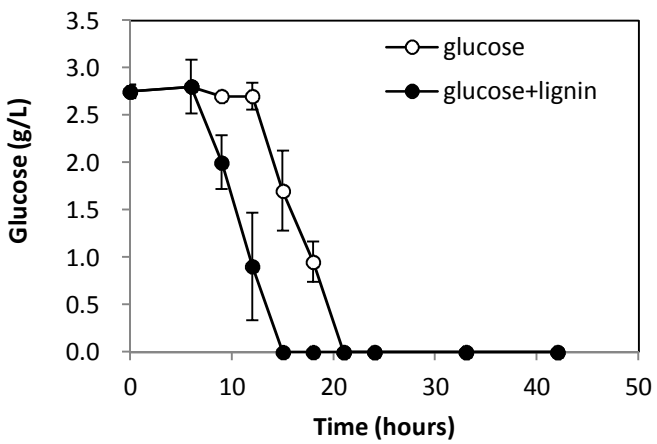
investigated in liquid LB and defined M9 glucose medium. In complex, nutrient-rich medium (e.g., LB medium), the addition of alkali lignin enhanced the cell growth by 80% and there is not significantly different maximum cell growth by the amounts of added lignin (Figure 7.3.2). The pH difference between the experiments was not observed. In the case of defined medium (e.g., M9 glucose medium), the growth rate of *Bacillus* sp. 275 was faster in lignin-added medium. The maximum cell growth was higher as shown in complex medium, but the difference of the maximum cell growth between lignin-added medium and glucose only medium was 17%. Faster glucose consumption and pH decrease were observed by the results of faster growth of *Bacillus* sp. 275 (Figure 7.3.1).

The change of alkali lignin was identified using gel permeation chromatography (GPC) (Figure 7.3.3). The peak shift of alkali lignin to left was detected in LB medium at initial medium pH 7. This result implies that the polymerization of lignin occurred. By contrast, no shift and shift to right were observed in M9 glucose medium at initial medium pH 7 and 6, respectively. As the growth of *Bacillus* sp. 275, the pH of LB medium was increased and the pH of M9 glucose medium was decreased. Polymerization and deploymerization of lignin during bacterial cell growth with lignin was detected in previous report[138]. Some bacteria conducted repolymerization to higher molecular weight species during biotransformation of lignin. In our results, repolymerization or deploymerization of lignin was related to the pH of medium. In general, the inhibition of cell growth was caused by the existence of lignin in culture medium. However, the growth of *Bacillus* sp. 275 was enhanced by lignin addition and the distribution of molecular weight of lignin was changed by the pH of growth medium.

(a)


OD at 600nm
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Time (hours)
glucose
glucose+lignin

(b)


Glucose (g/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Time (hours)
glucose
glucose+lignin

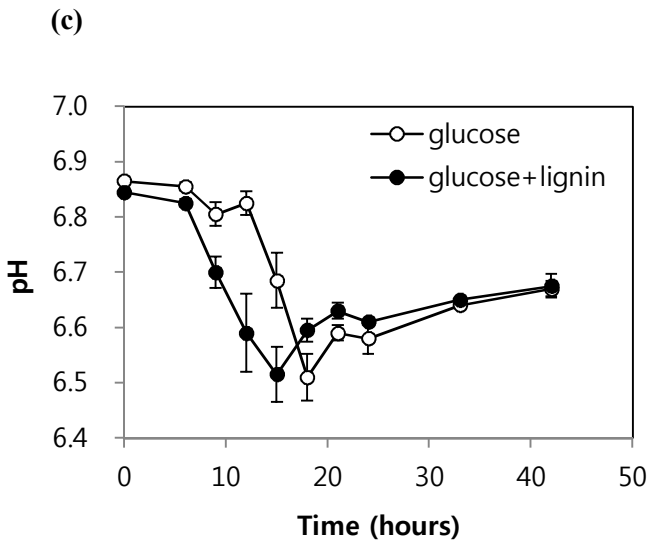


Figure 7.3.1 Effect of lignin addition on growth of *Bacillus* sp. 275 in defined medium. (a) OD at 600nm, (b) Glucose concentration, (c) pH

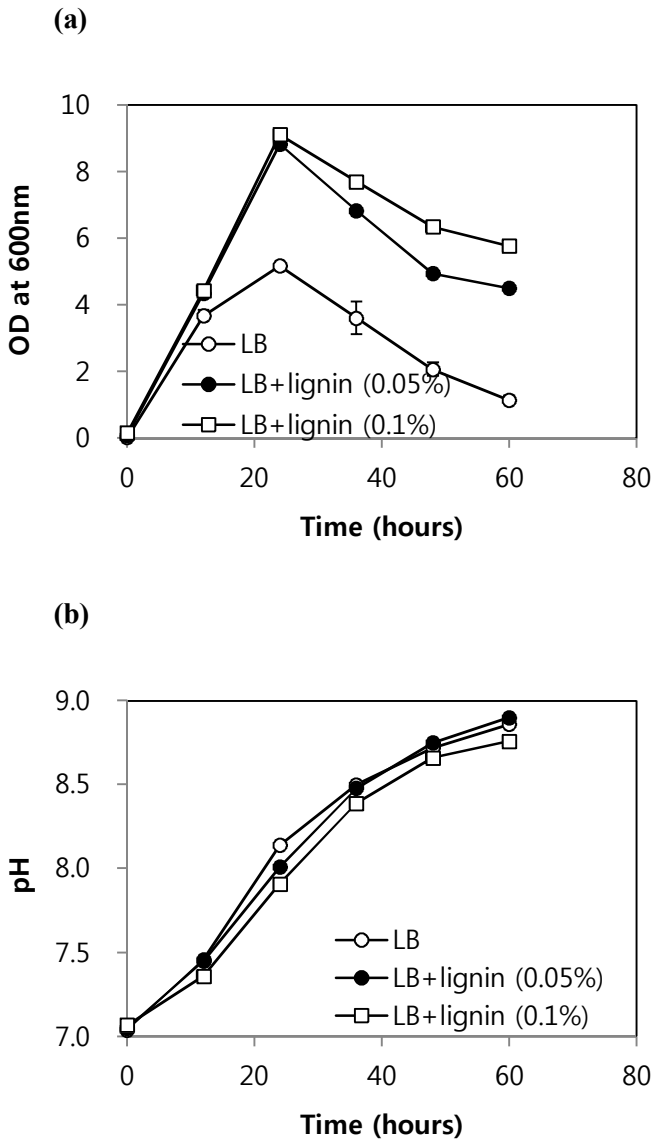
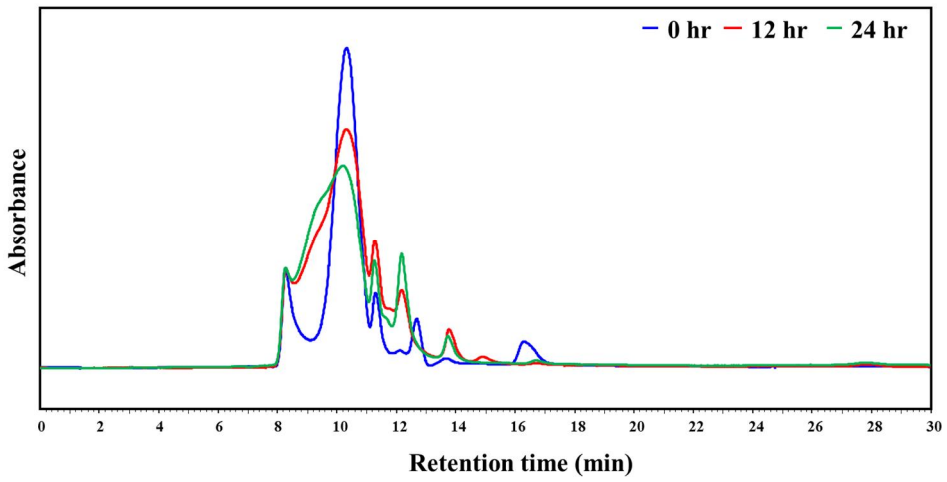
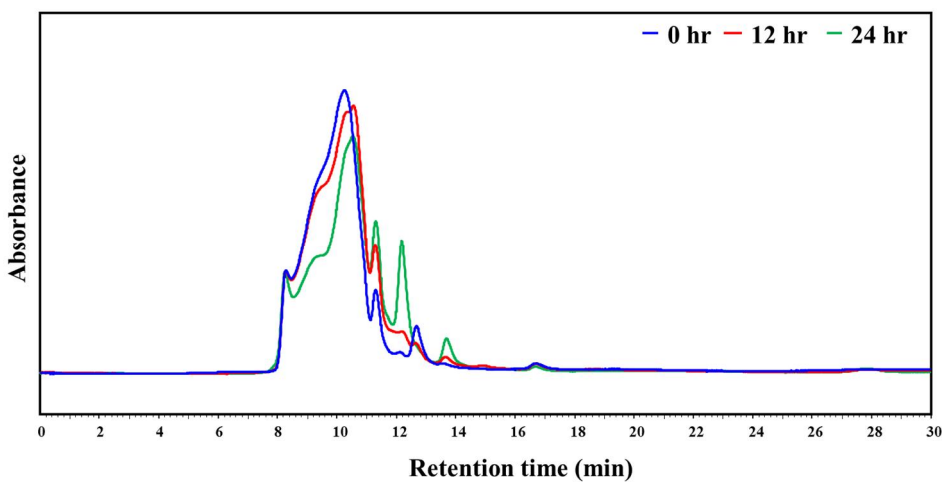


Figure 7.3.2 Effect of lignin addition on growth of *Bacillus* sp. 275 in complex medium. (a) OD at 600nm, (b) pH

(a)


0 hr
12 hr
24 hr
Absorbance
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Retention time (min)

(b)


0 hr
12 hr
24 hr
Absorbance
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Retention time (min)

(c)

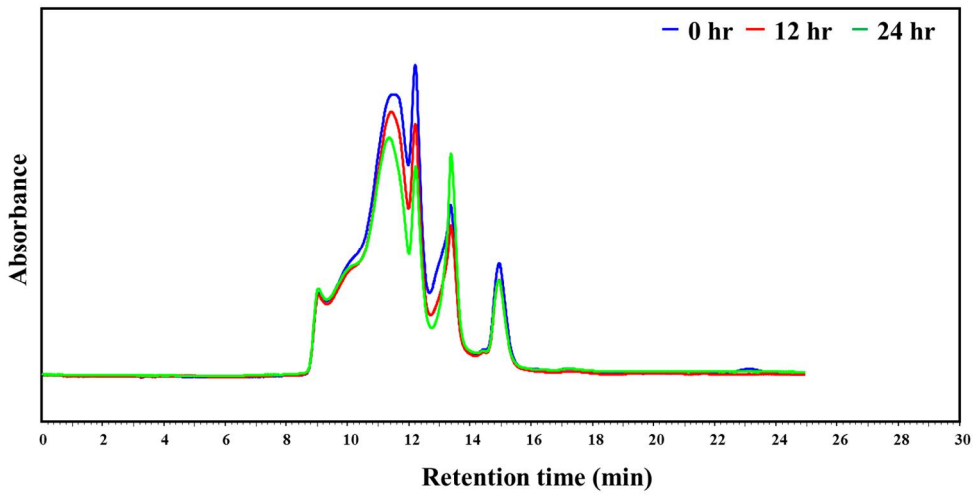


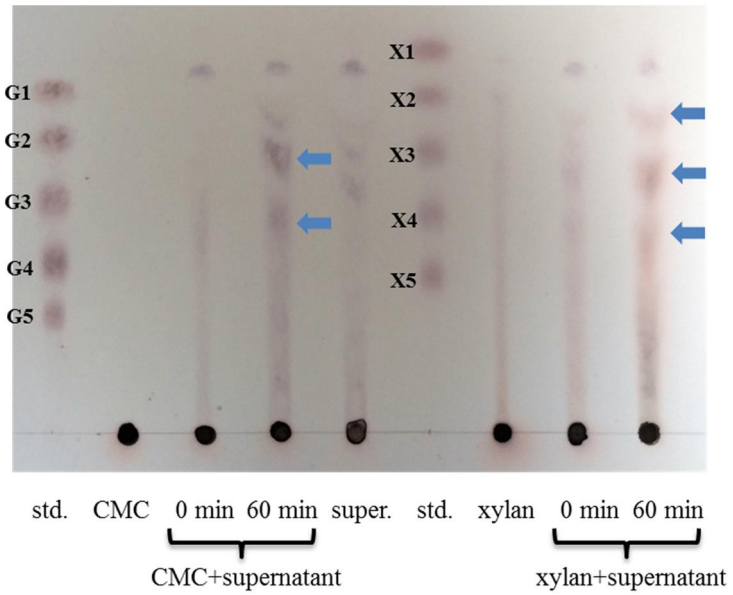
Figure 7.3.3 GPC profiles for supernatant of *Bacillus* sp. 275 grown in defined medium at initial pH 7 (a) and pH 6 (b), and complex medium at initial pH 7 (c).

7.4. Degradation of polysaccharides by extracellular enzymes

The supernatants of *Bacillus* sp. 275 included cellulose and xylan degrading enzymes because of degradation for high molecular weight of polysaccharide. The patterns of released CMC or xylan fragments in the reaction with cell-free supernatants of *Bacillus* sp. 275 grown in LB-based medium were observed by thin layer chromatography (TLC). Regarding to degradation of CMC, the putative spots of cellobiose (G2) and cellotriose (G3) were detected after 60 min of reaction with CMC and the supernatants of *Bacillus* sp. 275 grown both LB+CMC and LB+xylan. In contrast, the degradation products of xylan were different compared to those of CMC. The three spots estimated to be xylobiose (X2), xylotriose (X3) and xylotetraose (X4) were detected. These degradation patterns were shown more clearly in Figure 7.4.1. Furthermore, another spot (xylose) was also detected in Figure 7.4.1. These results seem to be attributed to higher concentration of extracellular proteins produced in LB+xylan medium.

The degradation products of CMC and xylan are more clearly revealed by HPLC analysis. As shown in Figure 7.4.2, the distinct peaks of cellobiose (G2) and cellotriose (G3) were detected as degradation products. Interestingly, glucose peak was not detected. This result suggest that *Bacillus* sp. 275 can be used cellulose as cellobiose form by degrading extracellular endoglucanase or exoglucanase because of absence of extracellular beta-glucosidase. However, this enzyme will exist in the intracellular protein for utilizing cellobiose. Regarding to xylan degradation, the peaks from xylose (X1) to xylopentaose (X5) were detected as the degradation products of xylan. To sum up, *Bacillus* sp. 275 exhibited cellulase and xylanase

(a)


G1
G2
G3
G4
G5
X1
X2
X3
X4
X5
std.
CMC
0 min
60 min
super.
std.
xylan
0 min
60 min
CMC+supernatant
xylan+supernatant

(b)

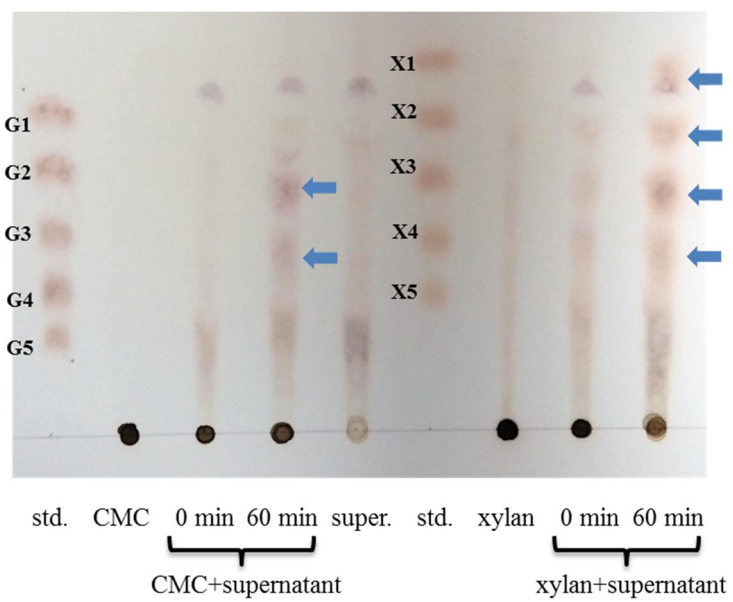
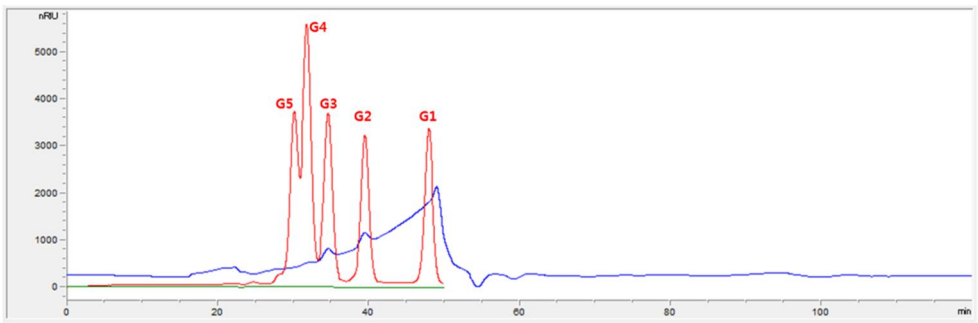

G1
G2
G3
G4
G5
X1
X2
X3
X4
X5
std.
CMC
0 min
60 min
super.
std.
xylan
0 min
60 min
CMC+supernatant
xylan+supernatant

Figure 7.4.1 Thin-layer chromatography analysis of hydrolysis products for CMC and xylan using supernatant of *Bacillus* sp. 275 grown in LB+CMC (A) and LB+xylan (B) medium. All samples were run against the cellooligosaccharide standards (glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4) and cellopentaose (G5)) and xylooligosaccharide standards (xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5)).

(a)



(b)

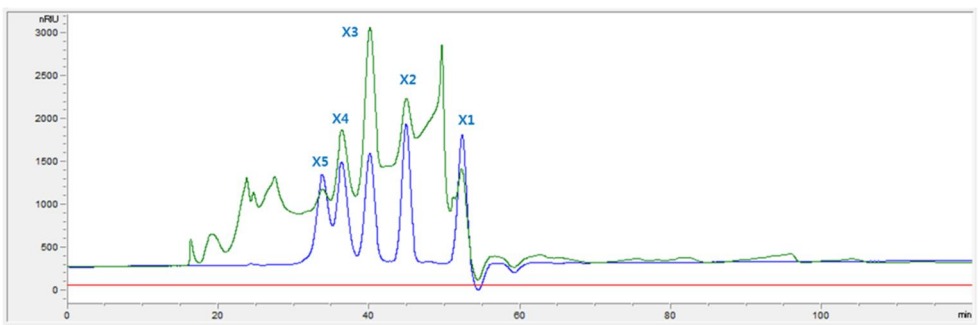


Figure7.4.2 HPLC analysis of the oligosaccharide products of CMC (a) and xylan (b). cellooligosaccharide and xylooligosaccharide standards are represented as G1~G5 and X1~X5, respectively.

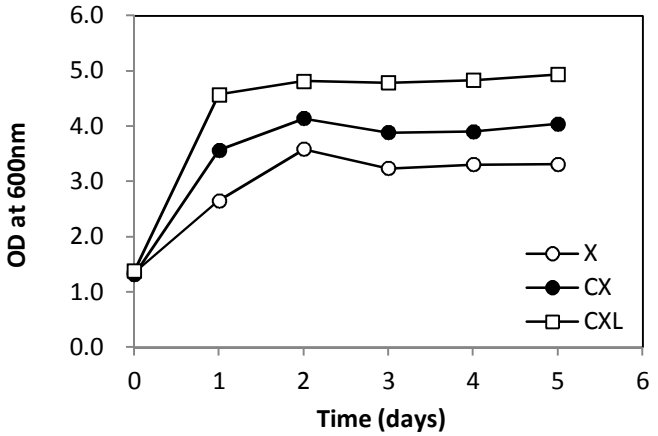
activities but there is no enzyme acting degradation of cellobiose extracellularly. In addition, the xylanase activity was higher than cellulase activity based on the degradation patterns (e.g., the numbers and amounts of oligosaccharides).

7.5. Growth dynamics of *Bacillus* sp. 275 in xylan-based media

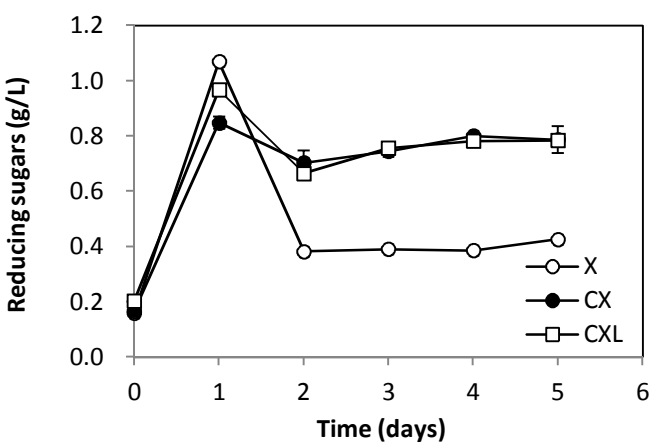
Unfortunately, *Bacillus* sp. 275 grows very slowly in M9 medium with cellulose or lignin as a sole carbon source. The fast growth was detected in M9 xylan medium. To investigate the effect of cellulose and lignin as a carbon source, CMC and lignin were added in xylan-based medium. Three kinds of growth medium were used in these experiments (e.g., X, CX and CXL).

The cell growth was increased by adding carbon sources such as cellulose and lignin (Figure 7.5.1). The amounts of reducing sugars in the supernatants were increased by extracellular polysaccharide-degrading proteins (e.g., cellulase and xylanase) during the first day. The decrease of reducing sugars was caused by utilization to cell growth. The activity of xylanase was higher than that of cellulase by 2 ~ 4 times. This result explains that slow growth of *Bacillus* sp. 275 in cellulose medium as a sole carbon source because of a weak cellulase activity. Interestingly, the results between the maximum cell growth and the xylanase activity were the complete opposite order. The maximum cell growth was observed in CXL medium, however, the lowest xylanase activity was detected. The data suggest that the amounts of xylanase produced by *Bacillus* sp. 275 were enough for

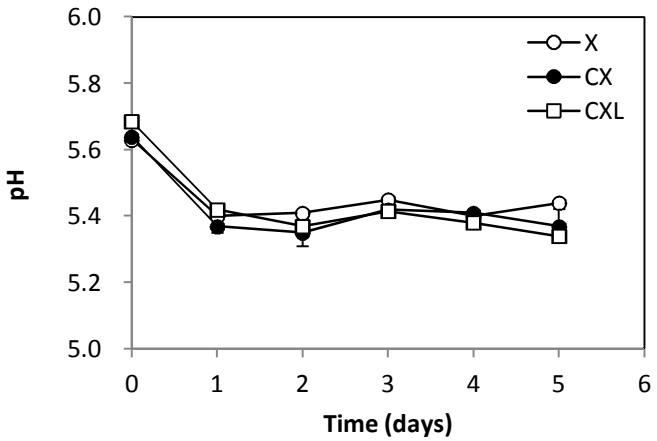
(a)


6.0
5.0
4.0
3.0
2.0
1.0
0.0
OD at 600nm
0
1
2
3
4
5
6
Time (days)
X
CX
CXL

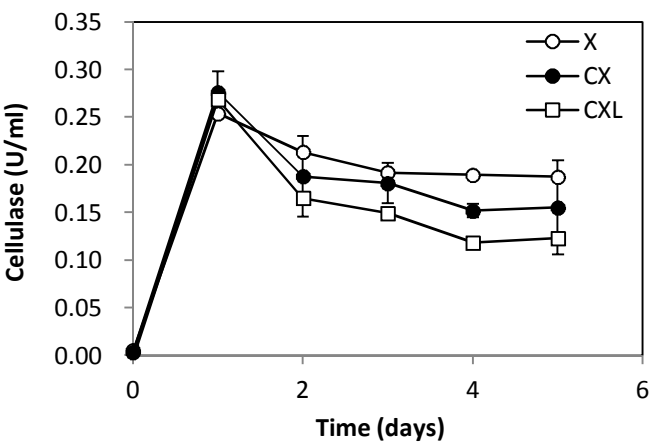
(b)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Reducing sugars (g/L)
0
1
2
3
4
5
6
Time (days)
X
CX
CXL

(c)


6.0
5.8
5.6
5.4
5.2
5.0
pH
0
1
2
3
4
5
6
Time (days)
X
CX
CXL

(d)


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Cellulase (U/ml)
0
2
4
6
Time (days)
X
CX
CXL

(e)

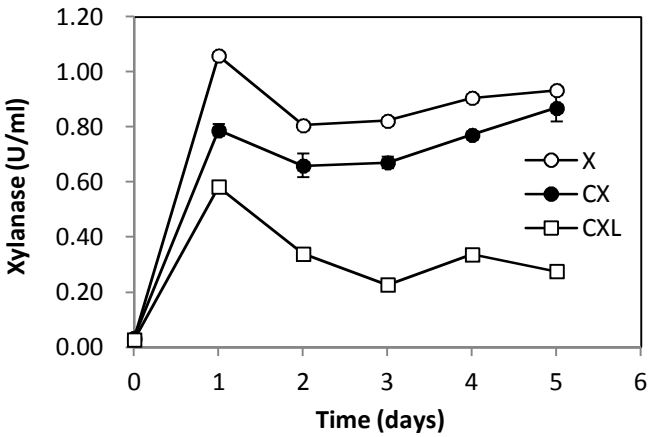


Figure7.5.1 Tine course of growth dynamics on xylan-based carbon sources. (a) OD at 600nm. (b) Reducing sugars in supernatant of cell broth. (c) pH. (d)-(e) The activities of cellulase and xylanase, respectively. Symbol: ○, xylan; ●, CMC and xylan; □, CMC, xylan and lignin.

utilizing of xylan to some extents. Additionally, there is also a possibility that the xylanase may have been adsorbed on surface of cellulose and lignin.

After 2 days of incubation, the experimental results did not change significantly. This result means that *Bacillus* sp. 275 utilized available some parts of substrates during 2 days, but do not use all parts of cellulose, xylan and lignin.

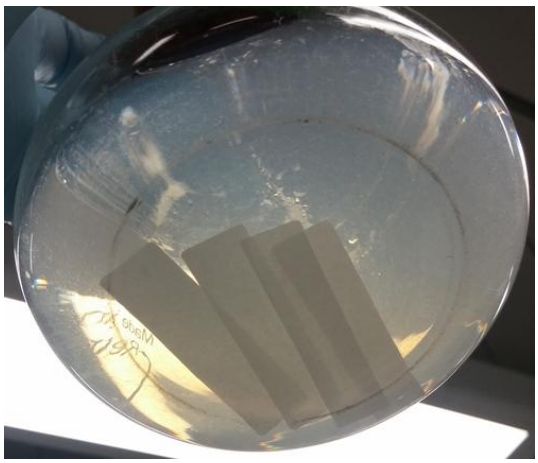
7.6. Filter paper degradation by *Bacillus* sp. 275

Filter paper degradation was studied by *Bacillus* sp. 275 in xylan-based medium supplemented with Whatman no.1 filter paper (Figure 7.6.1). Degradation of filter paper is commonly considered to have the ability to decompose cellulose by microorganisms.

After 2 days of incubation, filter paper was break into small pieces but the width of filter paper was not changed. Diverse shape of filter paper was observed after 4 days of incubation. From piece of almost same width compared to initial filter paper to small pieces of oval and circular shapes were appeared. After 11 days of incubation, only 2~3 small pieces of filter paper residues were remained in liquid medium. These figures suggest that *Bacillus* sp. 275 has an ability of degrading filter paper efficiently.

7.7. Conclusion

(a)



(b)

(c)

(d)

Figure 7.6.1 Filter paper degradation in CX medium supplemented with Whatman filter paper no. 1 (1 x 5cm strip x 4) after 0 day (a), 2 day (b), 4 day (c) and 11 days (d) of incubation.

The bacterial isolate, *Bacillus* sp. 275, showed cellulolytic, xylanolytic and lignolytic activities by extracellularly produced enzymes such as cellulase, xylanase, peroxidase and laccase. The cell growth was higher and faster when alkali lignin was added in the culture medium. Although *Bacillus* sp. 275 utilized alkali lignin as a carbon source, the deploymerization or repolymerization was occurred during cell growth by condition of culture medium. In the case of xylan degradation, xylose, xylobiose, xylotriose, xylotetraose and xylopentaose were detected as degradation products. Regarding to cellulose degradation, only cellobiose and cellotriose were observed. Despite the weak cellulase activity compared to xylanase, *Bacillus* sp. 275 can degrade filter paper vigorously in M9 xylan medium. As a result, *Bacillus* sp. 275 has a great potential for degrading or utilizing all major three components of lignocellulose.

Only a few bacterial isolates are reported to be able to degrade or utilize multicomponent of lignocellulose (Table 7.7.1). The researches related to these strains are at an early stage, such as measuring cell growth (i.e., increase of the optical density) or detecting lignocellulolytic enzymes. In addition, the activities of these bacterial lignocellulolytic enzymes are lower than those of fungi. However, lower enzyme activity can be overcome through relatively easy genetic manipulation tools compared to fungi.

Table 7.7.1 Bacterial strains for multicomponent utilization of lignocellulose

Bacterial strain	Relevant characteristics	Reference
Bacillus sp. CS-1	Partial degradation of cellulose, hemicellulose and lignin	Chang *et al.*[72]
Bacillus sp. 55S5	Production of cellulase and xylanase Modification of lignin	Maki *et al.*[132]
Bacillus sp. R2	Production of cellulase, xylanase and peroxidase	Khelil *et al.*[133]
Paenibacillus glucanolyticus	Utilization of cellulose, hemicellulose and lignin	Mathews *et al.*[139]
Streptomyces griseorubens C-5	Production of cellulase, laccase, peroxidase and xylanase	Xu *et al.*[57]
Bacillus sp. 275	Degradation of cellulose, xylan and lignin	This study

Chapter 8.

Overall discussion and further suggestions

Chapter 8. Overall discussion and further suggestions

Lignocellulosic biomass is one of the most plentiful renewable natural resources that can be used for the production of fuels and chemicals. Successful utilization and conversion of lignocellulose are expected through effective hydrolysis and fermentation using proper microbes with high lignocellulolytic activity. Among the components of lignocellulosic biomass - cellulose, hemicellulose and lignin, research has focused mainly on cellulose degradation and conversion to useful components, typically bioethanol[140, 141]. As a consequence, several second generation bioethanol facilities have been commercialized and operated[142]. However, the utilization of a whole biomass, which includes not only cellulose and hemicellulose but also lignin, is necessary to achieve economic feasibility[143]. Especially, lignin is well known to be recalcitrant for degradation although it is the most abundant natural aromatic polymer[144]. Generally, white-rot and brown-rot fungi are well known to mineralize lignin by producing extracellular lignolytic enzymes such as lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase. However, due to the difficulty of protein expression and fungal genetic manipulation, bacterial lignin degradation is receiving attention[145].

In this thesis, candidates for lignocellulolytic bacteria were isolated from various environmental sources and investigated their taxonomic distribution and cellulolytic, xylanolytic and lignolytic activities by isolation sites and compositions of isolation medium. A genomic analysis and lignocellulolytic enzyme activities of the finally selected bacterium (i.e., *Bacillus* sp. 275) were performed.

In chapter 4, the candidates of possible lignocellulose-degrading bacteria from environmental soil samples were successfully isolated and analyzed bacterial taxonomic distributions. Unlike the expected, lignocellulolytic bacteria were isolated both lignocellulose-rich and lignocellulose-rack samples. Regarding isolation media, cellulose-only media were effective to isolate bacteria exhibiting multienzymatic activities as well as cellulase activity. In contrast, lignin-containing medium was effective to isolating bacteria with lignolytic activity only, but not favorable to isolating bacteria exhibiting multienzymatic activities. In genus level, *Bacillus* and *Streptomyces* are isolated abundantly, which showed multienzymatic activities such as i) cellulase and xylanase or ii) cellulase, xylanase and ligninase. And the most abundant genus with lignolytic activity was *Burkholderia*. Based on these results, the isolation condition such as the sampling locations and the isolation medium was significant factors to efficiently isolating bacteria with lignocellulolytic activities.

In chapter 5, *Bacillus* sp. 275 was selected for degradation of lignocellulose among 89 isolates. As the first step, ten isolates exhibiting relatively high lignocellulolytic activities were selected based on the results on agar plates. Only three isolates including *Bacillus* sp. 275 were grown in the alkali lignin medium and the medium consisting of a combination of cellulose, xylan and lignin. Furthermore, the activities of secreted enzymes such as cellulase, xylanase and peroxidase (especially, lignin peroxidase) by *Bacillus* sp. 275 were higher than the other two isolates' activities.

In chapter 6, whole genome sequencing of *Bacillus* sp. 275, a potential lignocellulose-degrading bacterium was conducted and investigated into genomic

information. As the results, the complete genome of *Bacillus* sp. 275 consisted of one 4,045,581 bp chromosome and one 6,389 bp plasmid with 3,832 protein cording sequences (CDS), 86 tRNA genes, 27 rRNA genes and an average G+C content of 46.32%. Based on the results of the 16S rRNA sequence and the average nucleotide identity (ANI) values, the isolate was named as *Bacillus* sp. 275. The genes related to the degradation of lignocellulose were detected in the genome of *Bacillus* sp. 275 (Endoglucanase, β-glucanase, glucohydrolase, glucosidase, glycoside hydrolase 43 family protein (1,4-β-xylosidase), arabinoxylan arabinofuranohydrolase, glucuronoxylanase, α-N-arabinofuranoside, deferrochelatase (dye decolorizing peroxidase) and laccase). Moreover, *Bacillus* sp. 275 has the genes related to degradation of starch, mannan, galactoside and arabinan. These results imply that *Bacillus* sp. 275 has potentially wide range of uses in the degradation of polysaccharide in lignocellulosic biomasses.

In chapter 7, the degradation properties of lignocellulosic biomass were investigated by *Bacillus* sp. 275. *Bacillus* sp. 275 showed cellulolytic, xylanolytic and lignolytic activities by extracellularly produced enzymes (i.e., cellulase, xylanase, peroxidase and laccase). Xylose, xylobiose, xylotriose, xylotetraose and xylopentaose were detected as products of xylan degradation. In the case of cellulose degradation, only cellobiose and cellotriose were detected. Although the secreted cellulase activity was lower than xylanase, filter paper degradation occurred actively by *Bacillus* sp. 275. The cell growth with alkali lignin added to the medium was faster and increased than the control. Although *Bacillus* sp. 275 utilized alkali lignin as a carbon source, the deploymerization or repolymerization was occurred during cell growth by condition of culture medium. Based on these

results, *Bacillus* sp. 275 has a great potential for degrading or utilizing all major three components of lignocellulose.

In this thesis, bacteria with cellulolytic, xylanolytic and lignolytic activities were isolated from lignocellulose-rich (mountain) and lignocellulose-rare (wetland and mudflat) sites using isolation media containing the combination of lignocellulose components (cellulose, xylan and lignin) and the isolated bacteria were characterized by analyzing taxonomic ranks and lignocellulolytic activities. Most of the cellulolytic bacteria showed multienzymatic activities including xylanolytic activity. Lignin-deficient media were efficient in isolating bacteria exhibiting multienzymatic activities even including lignolytic activity, whereas a lignin-containing medium was favorable to isolating bacteria with lignolytic activity only. The genes encoding enzymes related to the degradation of cellulose, xylan and lignin were detected in the *Bacillus* sp. 275 genome. In fact, *Bacillus* sp. 275 showed the lignocellulolytic activities: cellulase, xylanase, laccase and peroxidase. In addition, the genes encoding glucosidases that hydrolyze starch, mannan, galactoside and arabinan were also found in the genome, implying that *Bacillus* sp. 275 has potentially wide range of uses in the degradation of polysaccharide in lignocellulosic biomasses.

The efficient utilization of abundant lignocellulose is essential for the economic feasibility and sustainability[146]. In order to achieve this objective, the utilization of all major components of lignocellulose (i.e., cellulose, hemicellulose and lignin) is necessary. *Bacillus* sp. 275 isolated from mud flat is expected to accomplish this purpose because of various lignocellulolytic enzyme activities. However, further researches are still required about application to real biomass.

Bibliography

1. Liguori, R., Ventorino, V., Pepe, O. & Faraco, V. Bioreactors for lignocellulose conversion into fermentable sugars for production of high added value products. *Appl. Microbiol. Biotechnol.* **100**, 597-611 (2016).

2. Lee, H.V., Hamid, S.B. & Zain, S.K. Conversion of lignocellulosic biomass to nanocellulose: structure and chemical process. *Sci. World J.* **2014**, 631013 (2014).

3. Park, S.H., Ong, R.G. & Sticklen, M. Strategies for the production of cell wall-deconstructing enzymes in lignocellulosic biomass and their utilization for biofuel production. *Plant biotechnol. J* **14**, 1329-1344 (2016).

4. Alfenore, S. & Molina-Jouve, C. Current status and future prospects of conversion of lignocellulosic resources to biofuels using yeasts and bacteria. *Process Biochem.* **51**, 1747-1756 (2016).

5. Zhao, H., Yu, H., Yuan, X., Piao, R., Li, H., Wang, X. & Cui, Z. Degradation of lignocelluloses in rice straw by BMC-9, a composite microbial system. *J. Microbiol. Biotechnol.* **24**, 585-724 (2014).

6. Ni, J. & Tokuda, G. Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnol. Adv.* **31**, 838-850 (2013).

7. Arevalo-Gallegos, A., Ahmad, Z., Asgher, M., Parra-Saldivar, R. & Iqbal, H.M. Lignocellulose: A sustainable material to produce value-added products with a zero waste approach-A review. *Int. J. Biol. Macromol.* **99**, 308-318 (2017).

8. Rosnow, J.J., Anderson, L.N., Nair, R.N., Baker, E.S. & Wright, A.T. Profiling microbial lignocellulose degradation and utilization by emergent omics technologies. *Crit. Rev. Biotechnol.* 1-15 (2016).

9. Capolupo, L. & Faraco, V. Green methods of lignocellulose pretreatment for biorefinery development. *Appl. Microbiol. Biotechnol.* **100**, 9451-9467 (2016).

10. Bilal, M., Asgher, M., Iqbal, H.M., Hu, H. & Zhang, X. Biotransformation of lignocellulosic materials into value-added products-A review. *Int. J. Biol. Macromol.* **98**, 447-458 (2017).

11. Meng, X. & Ragauskas, A.J. Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates. *Curr. Opin.Bbiotechnol.* **27**, 150-158 (2014).

12. Villares, A., Moreau, C., Bennati-Granier, C., Garajova, S., Foucat, L., Falourd, X., Saake, B., Berrin, J.G. & Cathala, B. Lytic polysaccharide monooxygenases disrupt the cellulose fibers structure. *Sci. Rep.* **7**, 40262

(2017).

13. Jeoh, T., Cardona, M.J., Karuna, N., Mudinoor, A.R. & Nill, J. Mechanistic kinetic models of enzymatic cellulose hydrolysis-a review. *Biotechnol. Bioeng.* **114**, 1369-1385 (2017).

14. Mathews, S.L., Pawlak, J. & Grunden, A.M. Bacterial biodegradation and bioconversion of industrial lignocellulosic streams. *Appl. Microbiol. Biotechnol.* **99**, 2939-2954 (2015).

15. Moreira, L.R. & Filho, E.X. Insights into the mechanism of enzymatic hydrolysis of xylan. *Appl. Microbiol. Biotechnol.* **100**, 5205-5214 (2016).

16. Ko, J.K., Um, Y., Park, Y.C., Seo, J.H. & Kim, K.H. Compounds inhibiting the bioconversion of hydrothermally pretreated lignocellulose. *Appl. Microbiol. Biotechnol.* **99**, 4201-4212 (2015).

17. Bugg, T.D., Ahmad, M., Hardiman, E.M. & Rahmanpour, R. Pathways for degradation of lignin in bacteria and fungi. *Nat. Prod. Rep.* **28**, 1883-1896 (2011).

18. Chen, Z. & Wan, C. Biological valorization strategies for converting lignin into fuels and chemicals. *Renewable and Sustainable Energy Rev.* **73**, 610-621 (2017).

19. Li, M., Pu, Y. & Ragauskas, A.J. Current understanding of the correlation of lignin structure with biomass recalcitrance. *Front. Chem.* **4**, 45 (2016).

20. Naseem, A., Tabasum, S., Zia, K.M., Zuber, M., Ali, M. & Noreen, A. Lignin-derivatives based polymers, blends and composites: A review. *Int. J. Biol. Macromol.* **93**, 296-313 (2016).

21. Cannatelli, M.D. & Ragauskas, A.J. Conversion of lignin into value-added materials and chemicals via laccase-assisted copolymerization. *Appl. Microbiol. Biotechnol.* **100**, 8685-8691 (2016).

22. Chen, R. & Dou, J. Biofuels and bio-based chemicals from lignocellulose: metabolic engineering strategies in strain development. *Biotechnol. lett.* **38**, 213-221 (2016).

23. Yu, C., Harrold, D.R., Claypool, J.T., Simmons, B.A., Singer, S.W., Simmons, C.W. & VanderGheynst, J.S. Nitrogen amendment of green waste impacts microbial community, enzyme secretion and potential for lignocellulose decomposition. *Process Biochem.* **52**, 214-222 (2017).

24. Cortes-Tolalpa, L., Jimenez, D.J., de Lima Brossi, M.J., Salles, J.F. & van Elsas, J.D. Different inocula produce distinctive microbial consortia with similar lignocellulose degradation capacity. *Appl. Microbiol. Biotechnol.* **100**, 7713-7725 (2016).

25. Jimenez, D.J., Korenblum, E. & van Elsas, J.D. Novel multispecies microbial consortia involved in lignocellulose and 5-hydroxymethylfurfural bioconversion. *Appl. Microbiol. Biotechnol.* **98**, 2789-2803 (2014).

26. Zuroff, T.R. & Curtis, W.R. Developing symbiotic consortia for lignocellulosic biofuel production. *Appl. Microbiol. Biotechnol.* **93**, 1423-1435 (2012).

27. Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, Y. & Wang, W. A novel lignin degradation bacterial consortium for efficient pulping. *Bioresour. Technol.* **139**, 113-119 (2013).

28. Ma, K. & Ruan, Z. Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi. *Bioresour. Technol.* **175**, 586-593 (2014).

29. Hu, J., Xue, Y., Guo, H., Gao, M.T., Li, J., Zhang, S. & Tsang, Y.F. Design and composition of synthetic fungal-bacterial microbial consortia that improve lignocellulolytic enzyme activity. *Bioresour. Technol.* **227**, 247-255 (2017).

30. Liao, J.C., Mi, L., Pontrelli, S. & Luo, S. Fuelling the future: microbial engineering for the production of sustainable biofuels. *Nat. Rev. Microbiol.* **14**, 288-304 (2016).

31. Frandsen, K.E. & Lo Leggio, L. Lytic polysaccharide monooxygenases: a crystallographer's view on a new class of biomass-degrading enzymes. *IUCrJ* **3**, 448-467 (2016).

32. Walton, P.H. & Davies, G.J. On the catalytic mechanisms of lytic polysaccharide monooxygenases. *Curr. Opin. Chem. Biol.* **31**, 195-207 (2016).

33. Vaaje-Kolstad, G., Forsberg, Z., Loose, J.S., Bissaro, B. & Eijsink, V.G. Structural diversity of lytic polysaccharide monooxygenases. *Curr. Opin. Struct. Biol.* **44**, 67-76 (2017).

34. Gardner, J.G., Crouch, L., Labourel, A., Forsberg, Z., Bukhman, Y.V., Vaaje-Kolstad, G., Gilbert, H.J. & Keating, D.H. Systems biology defines the biological significance of redox-active proteins during cellulose degradation in an aerobic bacterium. *Mol. Microbiol.* **94**, 1121-1133 (2014).

35. Span, E.A. & Marletta, M.A. The framework of polysaccharide monooxygenase structure and chemistry. *Curr. Opin. Struct. Biol.* **35**, 93-99 (2015).

36. Biely, P., Singh, S. & Puchart, V. Towards enzymatic breakdown of complex plant xylan structures: State of the art. *Biotechnol. Adv.* **34**, 1260-1274 (2016).

37. Collins, T., Gerday, C. & Feller, G. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **29**, 3-23 (2005).

38. de Gonzalo, G., Colpa, D.I., Habib, M.H. & Fraaije, M.W. Bacterial enzymes involved in lignin degradation. *J. Biotechnol.* **236**, 110-119 (2016).

39. Zhu, D., Zhang, P., Xie, C., Zhang, W., Sun, J., Qian, W.J. & Yang, B. Biodegradation of alkaline lignin by *Bacillus ligniniphilus* L1. *Biotechnol. Biofuels* **10**, 44 (2017).

40. Rashid, G.M., Taylor, C.R., Liu, Y., Zhang, X., Rea, D., Fulop, V. & Bugg, T.D. Identification of manganese superoxide dismutase from *Sphingobacterium* sp. T2 as a novel bacterial enzyme for lignin oxidation. *ACS Chem. Biol.* **10**, 2286-2294 (2015).

41. Mycroft, Z., Gomis, M., Mines, P., Law, P. & Bugg, T.D.H. Biocatalytic conversion of lignin to aromatic dicarboxylic acids in *Rhodococcus jostii* RHA1 by re-routing aromatic degradation pathways. *Green Chem.* **17**, 4974-4979 (2015).

42. McLeod, M.P., Warren, R.L., Hsiao, W.W., Araki, N., Myhre, M., Fernandes, C., Miyazawa, D., Wong, W., Lillquist, A.L., Wang, D., Dosanjh, M., Hara, H., Petrescu, A., Morin, R.D., Yang, G., Stott, J.M., Schein, J.E., Shin, H., Smailus, D., Siddiqui, A.S., Marra, M.A., Jones, S.J., Holt, R., Brinkman,

F.S., Miyauchi, K., Fukuda, M., Davies, J.E., Mohn, W.W. & Eltis, L.D. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 15582-15587 (2006).

43. Chen, H.P., Chow, M., Liu, C.C., Lau, A., Liu, J. & Eltis, L.D. Vanillin catabolism in *Rhodococcus jostii* RHA1. *Appl. Environ. Microbiol.* **78**, 586-588 (2012).

44. Ahmad, M., Roberts, J.N., Hardiman, E.M., Singh, R., Eltis, L.D. & Bugg, T.D. Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. *Biochemistry* **50**, 5096-5107 (2011).

45. Roberts, J.N., Singh, R., Grigg, J.C., Murphy, M.E., Bugg, T.D. & Eltis, L.D. Characterization of dye-decolorizing peroxidases from *Rhodococcus jostii* RHA1. *Biochemistry* **50**, 5108-5119 (2011).

46. Sainsbury, P.D., Hardiman, E.M., Ahmad, M., Otani, H., Seghezzi, N., Eltis, L.D. & Bugg, T.D. Breaking down lignin to high-value chemicals: the conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1. *ACS chem. Biol.* **8**, 2151-2156 (2013).

47. Rahmanpour, R. & Bugg, T.D. Assembly in vitro of *Rhodococcus jostii* RHA1 encapsulin and peroxidase DypB to form a nanocompartment. *FEBS*

J. **280**, 2097-2104 (2013).

48. Ravi, K., Garcia-Hidalgo, J., Gorwa-Grauslund, M.F. & Liden, G. Conversion of lignin model compounds by *Pseudomonas putida* KT2440 and isolates from compost. *Appl. Microbiol. Biotechnol.* **101**, 5059-5070 (2017).

49. Prabhakaran, M., Couger, M.B., Jackson, C.A., Weirick, T. & Fathepure, B.Z. Genome sequences of the lignin-degrading *Pseudomonas* sp. strain YS-1p and *Rhizobium* sp. strain YS-1r isolated from decaying wood. *Genome Announc.* **3** (2015).

50. Rahmanpour, R. & Bugg, T.D. Characterisation of Dyp-type peroxidases from *Pseudomonas fluorescens* Pf-5: Oxidation of Mn(II) and polymeric lignin by Dyp1B. *Arch. Biochem. Biophys.* **574**, 93-98 (2015).

51. Santos, A., Mendes, S., Brissos, V. & Martins, L.O. New dye-decolorizing peroxidases from *Bacillus subtilis* and *Pseudomonas putida* MET94: towards biotechnological applications. *Appl. Microbiol. Biotechnol.* **98**, 2053-2065 (2014).

52. Kalyani, D.C., Phugare, S.S., Shedbalkar, U.U. & Jadhav, J.P. Purification and characterization of a bacterial peroxidase from the isolated strain *Pseudomonas* sp. SUK1 and its application for textile dye decolorization.

Ann. Microbiol. **61**, 483-491 (2010).

53. Li, J., Liu, C., Li, B., Yuan, H., Yang, J. & Zheng, B. Identification and molecular characterization of a novel DyP-type peroxidase from *Pseudomonas aeruginosa* PKE117. *Appl. Biochem. Biotechnol.* **166**, 774-785 (2012).

54. Shui Yang, J., Ren Ni, J., Li Yuan, H. & Wang, E. Biodegradation of three different wood chips by *Pseudomonas* sp. PKE117. *Int. Biodeterior. Biodegrad.* **60**, 90-95 (2007).

55. Pinheiro, G.L., de Azevedo-Martins, A.C., Albano, R.M., de Souza, W. & Frases, S. Comprehensive analysis of the cellulolytic system reveals its potential for deconstruction of lignocellulosic biomass in a novel *Streptomyces* sp. *Appl. Microbiol. Biotechnol.* **101**, 301-319 (2017).

56. Feng, H., Sun, Y., Zhi, Y., Mao, L., Luo, Y., Wei, X. & Zhou, P. Lignocellulose degradation by the isolate of *Streptomyces griseorubens* JSD-1. *Funct. Integr. Genomics* **15**, 163-173 (2015).

57. Xu, J. & Yang, Q. Isolation and characterization of rice straw degrading *Streptomyces griseorubens* C-5. *Biodegradation* **21**, 107-116 (2010).

58. Niladevi, K.N., Sheejadevi, P.S. & Prema, P. Strategies for enhancing laccase

yield from *Streptomyces psammoticus* and its role in mediator-based decolorization of azo dyes. *Appl. Biochem. Biotechnol.***151**, 9-19 (2008).

59. Niladevi, K.N., Sukumaran, R.K. & Prema, P. Utilization of rice straw for laccase production by *Streptomyces psammoticus* in solid-state fermentation. *J. Ind. Microbiol. Biotechnol.* **34**, 665-674 (2007).

60. Davis, J.R. & Sello, J.K. Regulation of genes in *Streptomyces* bacteria required for catabolism of lignin-derived aromatic compounds. *Appl. Microbiol. Biotechnol.* **86**, 921-929 (2010).

61. Lu, L., Zeng, G., Fan, C., Ren, X., Wang, C., Zhao, Q., Zhang, J., Chen, M., Chen, A. & Jiang, M. Characterization of a laccase-like multicopper oxidase from newly isolated *Streptomyces* sp. C1 in agricultural waste compost and enzymatic decolorization of azo dyes. *Biochem. Eng. J.* **72**, 70-76 (2013).

62. Gottschalk, L.M., Bon, E.P. & Nobrega, R. Lignin peroxidase from *Streptomyces viridosporus* T7A: enzyme concentration using ultrafiltration. *Appl. Biochem. Biotechnol.* **147**, 23-32 (2008).

63. Zeng, J., Singh, D., Laskar, D.D. & Chen, S. Degradation of native wheat straw lignin by *Streptomyces viridosporus* T7A. *Int. J. Environ. Sci. Technol.* **10**, 165-174 (2012).

64. Sainsbury, P.D., Mineyeva, Y., Mycroft, Z. & Bugg, T.D. Chemical intervention in bacterial lignin degradation pathways: Development of selective inhibitors for intradiol and extradiol catechol dioxygenases. *Bioorg. Chem.* **60**, 102-109 (2015).

65. Zhao, C., Xie, S., Pu, Y., Zhang, R., Huang, F., Ragauskas, A.J. & Yuan, J.S. Synergistic enzymatic and microbial lignin conversion. *Green Chem.* **18**, 1306-1312 (2016).

66. Wei, Z., Zeng, G., Huang, F., Kosa, M., Huang, D. & Ragauskas, A.J. Bioconversion of oxygen-pretreated Kraft lignin to microbial lipid with oleaginous *Rhodococcus opacus* DSM 1069. *Green Chem.* **17**, 2784-2789 (2015).

67. Olibera E. R., Carnicero D., Jodra R., Miñambres B., García B., Abraham G. A., Gallardo A., Román J. S., García J. L., Naharro G. & Luengo J. M. Genetically engineered *Pseudomonas*: a factory of new bioplastics with broad applications. *Environ. Microbiol.* **3**, 612-618 (2011).

68. Davis, J.R., Goodwin, L., Teshima, H., Detter, C., Tapia, R., Han, C., Huntemann, M., Wei, C.L., Han, J., Chen, A., Kyrpides, N., Mavrommatis, K., Szeto, E., Markowitz, V., Ivanova, N., Mikhailova, N., Ovchinnikova, G., Pagani, I., Pati, A., Woyke, T., Pitluck, S., Peters, L., Nolan, M., Land, M. & Sello, J.K. Genome sequence of *Streptomyces viridosporus* strain T7A ATCC

39115, a lignin-degrading *Actinomycete*. *Genome announc.* **1** (2013).

69. Majumdar, S., Lukk, T., Solbiati, J.O., Bauer, S., Nair, S.K., Cronan, J.E. & Gerlt, J.A. Roles of small laccases from *Streptomyces* in lignin degradation. *Biochemistry* **53**, 4047-4058 (2014).

70. Nascimento, H.J. & Silva, J.G. Purification of lignin peroxidase isoforms from *Streptomyces viridosporus* T7A by hydrophobic based chromatographies. *World J. Microbiol. Biotechnol.* **24**, 1973-1975 (2008).

71. Raj, A., Reddy, M.M., Chandra, R., Purohit, H.J. & Kapley, A. Biodegradation of kraft-lignin by *Bacillus* sp. isolated from sludge of pulp and paper mill. *Biodegradation* **18**, 783-792 (2007).

72. Chang, Y.C., Choi, D., Takamizawa, K. & Kikuchi, S. Isolation of *Bacillus* sp. strains capable of decomposing alkali lignin and their application in combination with lactic acid bacteria for enhancing cellulase performance. *Bioresour. Technol.* **152**, 429-436 (2014).

73. Huang, X.F., Santhanam, N., Badri, D.V., Hunter, W.J., Manter, D.K., Decker, S.R., Vivanco, J.M. & Reardon, K.F. Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnol. Bioeng.* **110**, 1616-1626 (2013).

74. Raj, A., Reddy, M.M.K. & Chandra, R. Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading *Bacillus* sp. *J. Chem. Technol. Biotechnol.* **82**, 399-406 (2007).

75. Min, K., Gong, G., Woo, H.M., Kim, Y. & Um, Y. A dye-decolorizing peroxidase from *Bacillus subtilis* exhibiting substrate-dependent optimum temperature for dyes and beta-ether lignin dimer. *Sci. Rep.* **5**, 8245 (2015).

76. Shi, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Chen, Y. & Jing, Q. Biochemical investigation of kraft lignin degradation by *Pandoraea* sp. B-6 isolated from bamboo slips. *Bioprocess. Biosyst. Eng.* **36**, 1957-1965 (2013).

77. Chen, Y., Chai, L., Tang, C., Yang, Z., Zheng, Y., Shi, Y. & Zhang, H. Kraft lignin biodegradation by *Novosphingobium* sp. B-7 and analysis of the degradation process. *Bioresour. Technol.* **123**, 682-685 (2012).

78. Kumar, M., Singh, J., Singh, M.K., Singhal, A. & Thakur, I.S. Investigating the degradation process of kraft lignin by beta-proteobacterium, *Pandoraea* sp. ISTKB. *Environ. Sci. Pollut. Res. Int.* **22**, 15690-15702 (2015).

79. Deangelis, K.M., Sharma, D., Varney, R., Simmons, B., Isern, N.G., Markilllie, L.M., Nicora, C., Norbeck, A.D., Taylor, R.C., Aldrich, J.T. & Robinson, E.W. Evidence supporting dissimilatory and assimilatory lignin degradation in *Enterobacter lignolyticus* SCF1. *Front. Microbiol.* **4**, 280

(2013).

80. Deangelis, K.M., D'Haeseleer, P., Chivian, D., Fortney, J.L., Khudyakov, J., Simmons, B., Woo, H., Arkin, A.P., Davenport, K.W., Goodwin, L., Chen, A., Ivanova, N., Kyrpides, N.C., Mavromatis, K., Woyke, T. & Hazen, T.C. Complete genome sequence of "*Enterobacter lignolyticus*" SCF1. *Stand. Genomic Sci.* **5**, 69-85 (2011).

81. Woo, H.L., Utturkar, S., Klingeman, D., Simmons, B.A., DeAngelis, K.M., Brown, S.D. & Hazen, T.C. Draft genome sequence of the lignin-degrading *Burkholderia* sp. strain LIG30, isolated from wet tropical forest soil. *Genome Announc.* **2** (2014).

82. Brown, M.E., Barros, T. & Chang, M.C. Identification and characterization of a multifunctional dye peroxidase from a lignin-reactive bacterium. *ACS Chem. Biol.* **7**, 2074-2081 (2012).

83. Strachan, C.R., Singh, R., VanInsberghe, D., Ievdokymenko, K., Budwill, K., Mohn, W.W., Eltis, L.D. & Hallam, S.J. Metagenomic scaffolds enable combinatorial lignin transformation. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 10143-10148 (2014).

84. Gall, D.L., Ralph, J., Donohue, T.J. & Noguera, D.R. A group of sequence-related sphingomonad enzymes catalyzes cleavage of beta-aryl ether

linkages in lignin beta-guaiacyl and beta-syringyl ether dimers. *Environ. Sci. Technol.* **48**, 12454–12463 (2014).

85. Picart, P., Muller, C., Mottweiler, J., Wiermans, L., Bolm, C., Dominguez de Maria, P. & Schallmey, A. From gene towards selective biomass valorization: bacterial beta-etherases with catalytic activity on lignin-like polymers. *ChemSusChem* **7**, 3164–3171 (2014).

86. Song, H.T., Gao, Y., Yang, Y.M., Xiao, W.J., Liu, S.H., Xia, W.C., Liu, Z.L., Yi, L. & Jiang, Z.B. Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates. *Bioresour. Technol.* **219**, 710-715 (2016).

87. Hu, J., Arantes, V. & Saddler, J.N. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnol. Biofuels* **4**, 36 (2011).

88. Davidi, L., Morais, S., Artzi, L., Knop, D., Hadar, Y., Arfi, Y. & Bayer, E.A. Toward combined delignification and saccharification of wheat straw by a laccase-containing designer cellulosome. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 10854-10859 (2016).

89. Tian, J.H., Pourcher, A.M., Bouchez, T., Gelhaye, E. & Peu, P. Occurrence of

lignin degradation genotypes and phenotypes among prokaryotes. *Appl. Microbiol. Biotechnol.* **98**, 9527-9544 (2014).

90. Picart, P., Wiermans, L., Pérez-Sánchez, M., Grande, P.M., Schallmey, A. & Domínguez de María, P. Assessing lignin types to screen novel biomass-degrading microbial strains: synthetic lignin as useful carbon source. *ACS Sustainable Chem. Eng.* **4**, 651-655 (2016).

91. Zhu, N., Yang, J., Ji, L., Liu, J., Yang, Y. & Yuan, H. Metagenomic and metaproteomic analyses of a corn stover-adapted microbial consortium EMSD5 reveal its taxonomic and enzymatic basis for degrading lignocellulose. *Biotechnol. Biofuels* **9**, 243 (2016).

92. Montella, S., Amore, A. & Faraco, V. Metagenomics for the development of new biocatalysts to advance lignocellulose saccharification for bioeconomic development. *Crit. Rev. Biotechnol.* **36**, 998-1009 (2016).

93. Armstrong, Z., Mewis, K., Strachan, C. & Hallam, S.J. Biocatalysts for biomass deconstruction from environmental genomics. *Curr. Opin. Chem. Biol.* **29**, 18-25 (2015).

94. Schofield, M.M. & Sherman, D.H. Meta-omic characterization of prokaryotic gene clusters for natural product biosynthesis. *Curr. Opin. Biotechnol.* **24**, 1151-1158 (2013).

95. Warnecke, F., Luginbuhl, P., Ivanova, N., Ghassemian, M., Richardson, T.H., Stege, J.T., Cayouette, M., McHardy, A.C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S.G., Podar, M., Martin, H.G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N.C., Matson, E.G., Ottesen, E.A., Zhang, X., Hernandez, M., Murillo, C., Acosta, L.G., Rigoutsos, I., Tamayo, G., Green, B.D., Chang, C., Rubin, E.M., Mathur, E.J., Robertson, D.E., Hugenholtz, P. & Leadbetter, J.R. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**, 560-565 (2007).

96. Picart, P., de Maria, P.D. & Schallmey, A. From gene to biorefinery: microbial beta-etherases as promising biocatalysts for lignin valorization. *Front. Microbiol.* **6**, 916 (2015).

97. Fache, M., Boutevin, B. & Caillol, S. Vanillin production from lignin and its use as a renewable chemical. *ACS Sustainable Chem. Eng.* **4**, 35-46 (2016).

98. Sana, B., Chia, K.H., Raghavan, S.S., Ramalingam, B., Nagarajan, N., Seayad, J. & Ghadessy, F.J. Development of a genetically programed vanillin-sensing bacterium for high-throughput screening of lignin-degrading enzyme libraries. *Biotechnol. Biofuels* **10**, 32 (2017).

99. Zamzuri, N.A., Abd-Aziz, S., Rahim, R.A., Phang, L.Y., Alitheen, N.B. & Maeda, T. A rapid colorimetric screening method for vanillic acid and

vanillin-producing bacterial strains. *J. Appl. Microbiol.* **116**, 903-910 (2014).

100. Sato, Y., Moriuchi, H., Hishiyama, S., Otsuka, Y., Oshima, K., Kasai, D., Nakamura, M., Ohara, S., Katayama, Y., Fukuda, M. & Masai, E. Identification of three alcohol dehydrogenase genes involved in the stereospecific catabolism of arylglycerol-beta-aryl ether by *Sphingobium* sp. strain SYK-6. *Appl. Environ. Microbiol.* **75**, 5195-5201 (2009).

101. Yoshikata, T., Suzuki, K., Kamimura, N., Namiki, M., Hishiyama, S., Araki, T., Kasai, D., Otsuka, Y., Nakamura, M., Fukuda, M., Katayama, Y. & Masai, E. A three-component O-demethylase system essential for catabolism of a lignin-derived biphenyl compound in *Sphingobium* sp. strain SYK-6. *Appl. Environ. Microbiol.* **80**, 7142-7143 (2014).

102. Barry, K.P., Ngu, A., Cohn, E.F., Cote, J.M., Maxwell Burroughs, A., Gerbino, J.P. & Taylor, E.A. Exploring allosteric activation of LigAB from *Sphingobium* sp. strain SYK-6 through kinetics, mutagenesis and computational studies. *Arch. Biochem. Biophys.* **567**, 35-45 (2015).

103. Masai, E., Katayama, Y. & Fukuda, M. Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds. *Biosci. Biotechnol., Biochem.* **71**, 1-15 (2007).

104. Teather, R.M. & Wood, P.J. Use of Congo red polysaccharide interactions in

enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **43**, 777-780 (1982).

105. Saini, J.K., Arti & Tewari, L. Simultaneous isolation and screening of cellulolytic bacteria: selection of efficient medium. *J. Pure Appl. Microbiol.* **6**, 1339-1344 (2012).

106. Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S. & Chun, J. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **62**, 716-721 (2012).

107. Kumar, S., Stecher, G. & Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **33**, 1870-1874 (2016).

108. Woo, H.L., Hazen, T.C., Simmons, B.A. & DeAngelis, K.M. Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. *Syst. Appl. Microbiol.* **37**, 60-67 (2014).

109. Ventorino, V., Aliberti, A., Faraco, V., Robertiello, A., Giacobbe, S., Ercolini, D., Amore, A., Fagnano, M. & Pepe, O. Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-

degrading bacteria for industrial biotechnological application. *Sci. Rep.* **5**, 8161 (2015).

110. Mathews, S.L., Pawlak, J. & Grunden, A.M. Bacterial biodegradation and bioconversion of industrial lignocellulosic streams. *Appl. Microbiol. Biotechnol.* **99**, 2939-2954 (2015).

111. Mathews, S.L., Grunden, A.M. & Pawlak, J. Degradation of lignocellulose and lignin by *Paenibacillus glucanolyticus*. *Int. Biodeterior. Biodegrad.* **110**, 79-86 (2016).

112. Huang, X.F., Santhanam, N., Badri, D.V., Hunter, W.J., Manter, D.K., Decker, S.R., Vivanco, J.M. & Reardon, K.F. Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnol. Bioeng.* **110**, 1616-1626 (2013).

113. Chang, Y.C., Choi, D., Takamizawa, K. & Kikuchi, S. Isolation of *Bacillus* sp. strains capable of decomposing alkali lignin and their application in combination with lactic acid bacteria for enhancing cellulase performance. *Bioresour. Technol.* **152**, 429-436 (2014).

114. Bandounas, L., Wierckx, N.J., de Winde, J.H. & Ruijssenaars, H.J. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotechnol.* **11**, 94 (2011).

115. Bugg, T.D., Ahmad, M., Hardiman, E.M. & Singh, R. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr. Opin. Biotechnol.* **22**, 394-400 (2011).

116. Maki, M.L., Idrees, A., Leung, K.T. & Qin, W. Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass. *J. Mol. Microbiol. Biotechnol.* **22**, 156-166 (2012).

117. Sizova, M.V., Izquierdo, J.A., Panikov, N.S. & Lynd, L.R. Cellulose- and xylan-degrading thermophilic anaerobic bacteria from biocompost. *Appl. Environ. Microbiol.* **77**, 2282-2291 (2011).

118. Deangelis, K.M., Sharma, D., Varney, R., Simmons, B., Isern, N.G., Markilllie, L.M., Nicora, C., Norbeck, A.D., Taylor, R.C., Aldrich, J.T. & Robinson, E.W. Evidence supporting dissimilatory and assimilatory lignin degradation in *Enterobacter lignolyticus* SCF1. *Front. Microbiol.* **4**, 280 (2013).

119. Roth, S. & Spiess, A.C. Laccases for biorefinery applications: a critical review on challenges and perspectives. *Bioprocess. Biosyst. Eng.* **38**, 2285-2313 (2015).

120. Latorre, J.D., Hernandez-Velasco, X., Kuttappan, V.A., Wolfenden, R.E., Vicente, J.L., Wolfenden, A.D., Bielke, L.R., Prado-Rebolledo, O.F., Morales,

E., Hargis, B.M. & Tellez, G. Selection of *Bacillus* spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and *Clostridium perfringens* proliferation using an *in vitro* digestive model in different poultry diets. *Front. Vet. Sci.* **2**, 25 (2015).

121. Min, K., Gong, G., Woo, H.M., Kim, Y. & Um, Y. A dye-decolorizing peroxidase from *Bacillus subtilis* exhibiting substrate-dependent optimum temperature for dyes and beta-ether lignin dimer. *Sci. Rep.* **5**, 8245 (2015).

122. Feng, H., Sun, Y., Zhi, Y., Mao, L., Luo, Y., Wei, X. & Zhou, P. Lignocellulose degradation by the isolate of *Streptomyces griseorubens* JSD-1. *Funct. Integr. Genomics* **15**, 163-173 (2015).

123. Majumdar, S., Lukk, T., Solbiati, J.O., Bauer, S., Nair, S.K., Cronan, J.E. & Gerlt, J.A. Roles of small laccases from *Streptomyces* in lignin degradation. *Biochemistry* **53**, 4047-4058 (2014).

124. Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.* **36**, 1251-1275 (1992).

125. Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachua, D. & Vardon, D.R.

Opportunities and challenges in biological lignin valorization. *Curr. Opin. Biotechnol.* **42**, 40-53 (2016).

126. Woo, H.L., Utturkar, S., Klingeman, D., Simmons, B.A., DeAngelis, K.M., Brown, S.D. & Hazen, T.C. Draft genome sequence of the lignin-degrading *Burkholderia* sp. strain LIG30, isolated from wet tropical forest soil. *Genome announc.* **2**, e00637-00614 (2014).

127. Akita, H., Kimura, Z., Mohd Yusoff, M.Z., Nakashima, N. & Hoshino, T. Isolation and characterization of *Burkholderia* sp. strain CCA53 exhibiting ligninolytic potential. *Springerplus* **5**, 596 (2016).

128. Haft, R.J., Gardner, J.G. & Keating, D.H. Quantitative colorimetric measurement of cellulose degradation under microbial culture conditions. *Appl. Microbiol. Biotechnol.* **94**, 223-229 (2012).

129. Zhu, D., Tanabe, S.H., Xie, C., Honda, D., Sun, J. & Ai, L. *Bacillus ligniniphilus* sp. nov., an alkaliphilic and halotolerant bacterium isolated from sediments of the South China Sea. *Int. J. Syst. Evol. Microbiol.* **64**, 1712-1717 (2014).

130. Huang, X.F., Santhanam, N., Badri, D.V., Hunter, W.J., Manter, D.K., Decker, S.R., Vivanco, J.M. & Reardon, K.F. Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnol. Bioeng.* **110**, 1616-1626

(2013).

131. Bandounas, L., Wierckx, N.J., de Winde, J.H. & Ruijssenaars, H.J. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotechnol.* **11**, 94 (2011).

132. Maki, M.L., Idrees, A., Leung, K.T. & Qin, W. Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass. *J. Mol. Microbiol. Biotechnol.* **22**, 156-166 (2012).

133. Khelil, O., Choubane, S. & Cheba, B.A. Polyphenols content of spent coffee grounds subjected to physico-chemical pretreatments influences lignocellulolytic enzymes production by *Bacillus* sp. R2. *Bioresour. Technol.* **211**, 769-773 (2016).

134. Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S. & Chun, J. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **62**, 716-721 (2012).

135. Dunlap, C.A., Kim, S.J., Kwon, S.W. & Rooney, A.P. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp *plantarum* and '*Bacillus*

oryzicola' are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. *Int. .J Syst. Evol. Microbiol.* **66**, 1212-1217 (2016).

136. Tatusov, R.L., Galperin, M.Y., Natale, D.A. & Koonin, E.V. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **28**, 33-36 (2000).

137. Kim, S.Y., Lee, S.Y., Weon, H.Y., Sang, M.K. & Song, J. Complete genome sequence of *Bacillus velezensis* M75, a biocontrol agent against fungal plant pathogens, isolated from cotton waste. *J. Biotechnol.* **241**, 112-115 (2017).

138. Taylor, C.R., Hardiman, E.M., Ahmad, M., Sainsbury, P.D., Norris, P.R. & Bugg, T.D. Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. *J. Appl. Microbiol.* **113**, 521-530 (2012).

139. Mathews, S.L., Pawlak, J.J. & Grunden, A.M. Isolation of *Paenibacillus glucanolyticus* from pulp mill sources with potential to deconstruct pulping waste. *Bioresour. Technol.* **164**, 100-105 (2014).

140. Jeon, H., Kang, K.-E., Jeong, J.-S., Gong, G., Choi, J.-W., Abimanyu, H., Ahn, B.S., Suh, D.-J. & Choi, G.-W. Production of anhydrous ethanol using oil palm empty fruit bunch in a pilot plant. *Biomass Bioenergy* **67**, 99-107 (2014).

141. Han, M., Kim, Y., Cho, W.S., Choi, G.W. & Chung, B.W. An integrated process for continuous cellulosic bioethanol production. *Korean J. Chem. Eng.* **33**, 223-229 (2015).

142. Mawhood, R., Gazis, E., de Jong, S., Hoefnagels, R. & Slade, R. Production pathways for renewable jet fuel: a review of commercialization status and future prospects. *Biofuels, Bioprod. Biorefin.* **10**, 462-484 (2016).

143. Perez, J., Munoz-Dorado, J., de la Rubia, T. & Martinez, J. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int Microbiol* **5**, 53-63 (2002).

144. Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachua, D. & Vardon, D.R. Opportunities and challenges in biological lignin valorization. *Curr. Opin. Biotechnol.* **42**, 40-53 (2016).

145. Bugg, T.D., Ahmad, M., Hardiman, E.M. & Singh, R. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr. Opin. Biotechnol.* **22**, 394-400 (2011).

146. Ma, R., Xu, Y. & Zhang, X. Catalytic oxidation of biorefinery lignin to value-added chemicals to support sustainable biofuel production. *ChemSusChem* **8**, 24-51 (2014).

Appendix

Table S1. Identification of all bacterial isolates

Bacterial isolates	Identification (Top-hit taxon)	16S rRNA gene similarity (%)
111	*Bacillus anthracis*	99.77
112	*Bacillus mycoides*	99.79
113	*Streptomyces mirabilis*	99.15
114	*Burkholeria tropica*	97.53
116	*Streptacidiphilus rugosus*	99.42
117	*Kitasatospora cystarginea*	98.29
121	*Bacillus acidiceler*	99.31
122	*Filimonas lacunae*	91.60
123	*Bacillus aryabhattai*	99.86
131	*Burkholeria fungorum*	98.50
132	*Streptomyces olivochromogenes*	99.71
133	*Bacillus acidiceler*	99.93
135	*Dyella soli*	97.96
141	*Bacillus acidiceler*	98.96
271	*Bacillus drentensis*	99.50
272	*Bacillus aryabhattai*	99.65
273	*Bacillus megaterium*	99.79
274	*Bacillus anthracis*	99.62
275	*bacillus siamensis*	99.86
311	*Bacillus anthracis*	99.54
312	*Burkholderia unamae*	98.50
313	*Citrobacter freundii*	99.79
314	*Phyllobacterium myrsinacearum*	99.12
315	*Burkholderia stabilis*	99.71
321	*Bacillus thuringiensis*	99.86
331	*Streptomyces indigoferus*	99.08
333	*Bacillus anthracis*	99.62
341	*Bacillus aryabhattai*	99.93

342	*Luteibacter anthropi*	98.73
343	*Enterobacter cancerogenus*	99.57
402	*Streptomyces canarius*	99.21
411	*Rhodanobacter ginsengisoli*	98.08
412	*Bacillus aryabhattai*	99.79
413	*Bacillus aryabhattai*	99.72
414	*Streptomyces durhamensis*	98.85
421	*Bacillus pseudomycoides*	98.74
422	*Bacillus thuringiensis*	99.86
423	*Bacillus thuringiensis*	99.86
424	*Bacillus pseudomycoides*	100
431	*Bacillus acidiceler*	99.93
432	*Bacillus aryabhattai*	99.93
433	*Streptomyces vinaceus*	99.78
434	*Bacillus koreensis*	99.93
435	*Bacillus muralis*	98.12
436	*Streptomyces phaeoluteigriseus*	99.14
441	*Caulobacter segnis*	98.32
442	*Bacillus anthracis*	99.85
443	*Bacillus thuringiensis*	99.72
501	*Enterobacter asburiae*	99.14
502	*Enterobacter cancerogenus*	99.15
511	*Streptomyces xanthocidicus*	98.79
512	*Bacillus acidiceler*	99.79
513	*Bacillus pseudomycoides*	100
514	*Bacillus subtilis* subsp. *inaquosorum*	99.91
515	*Caulobacter vibrioides*	99.41
516	*Bacillus aryabhattai*	99.69
517	*Streptomyces bungoensis*	98.81
521	*Bacillus anthracis*	99.92
522	*Bacillus aryabhattai*	99.65
523	*Bacillus thuringiensis*	99.93
531	*Bacillus acidiceler*	99.79
532	*Bacillus aryabhattai*	99.86
534	*Bacillus koreensis*	99.93

535	*Bacillus thuringiensis*	99.86
542	*Bacillus thuringiensis*	99.79
543	*Bacillus anthracis*	99.85
592	*Klebsiella oxytoca*	99.09
593	*Klebsiella oxytoca*	99.37
594	*Klebsiella michiganensis*	99.48
611	*Mesorhizobium gobiense*	99.26
614	*Mesorhizobium huakuii*	99.71
615	*Streptomyces olivochromogenes*	97.62
621	*Pseudomonas mosselii*	98.18
623	*Chitinophaga eiseniae*	96.61
624	*Pseudomonas vranovensis*	98.94
652	*Dyella soli*	98.52
653	*Pseudomonas koreensis*	99.30
713	*Bacillus mycoides*	99.59
722	*Pseudomonas plecoglossicida*	99.30
725	*Serratia glossinae*	99.44
753	*Burkholderia ferrariae*	97.18
754	*Variovorax boronicumulans*	98.67
755	*Xanthomonas gardneri*	97.49
812	*Burkholderia terrae*	98.60
815	*Burkholderia ubonensis*	98.78
851	*Burkholderia cenocepacia*	99.14
853	*Burkholderia pyrrocinia*	99.29
854	*Burkholderia pyrrocinia*	99.36
856	*Kitasatospora griseola*	98.23

초 록

이 연구의 목적은 리그노셀룰로오스 분해 박테리아를 환경으로부터 분리하고, 리그노셀룰로오스 관련 분해 활성을 연구하는 것이다. 또한, 선택된 균주인 *Bacillus* sp. 275의 리그노셀룰로오스 분해 효소의 활성 및 전체 유전체 분석에 관한 연구도 수행하였다. 이 연구에서는 다양한 환경 토양 샘플과 분리 배지를 사용하여 여러 종류의 분리 균주를 획득하였다. 또한, 셀룰로오스 및 자일란에서의 세포 성장, 효소 활성 및 분해 산물을 분석하였다.

첫 번째로, 환경 토양으로부터 미생물을 분리하고 셀룰로오스, 자일란 및 리그닌 분해 활성 및 분류학적 분포를 연구하였다. 최종적으로, 89개의 균주가 환경 시료로부터 분리되었다. 리그노셀룰로오스 박테리아는 리그노셀룰로오스가 풍부한 토양뿐만 아니라 리그노셀룰로오스가 희박한 토양 샘플에서도 분리되었다. 모든 균주는 4가지 계통 (*Firmicutes*, *Proteobacteria*, *Actinobacteria* 및 *Bacteroidetes*)에 포함되었다. *Bacillus*와 *Streptomyces*속은 70% 이상의 박테리아가 복합적 효소 활성을 나타내었고, 리그닌 분해 활성이 가장 풍부한 속은 *Burkholderia*였다. 리그닌 함유 배지는 리그닌 분해 활성만 존재하는 박테리아를 분리하는데 효과적이었지만, 복합적 효소 활성을 보이는 박테리아를 분리하는 것에는 유리하지 않았다.

두 번째로, 높은 리그노셀룰로오스 분해 활성을 나타내는 박테리아

균주를 세포의 성장 및 효소 활성을 통하여 선택하였다. *Bacillus* sp. 275는 리그닌 배지에서 성장이 가능하였고, 123 균주 (*Bacillus aryabhattai*) 및 414 균주 (*Streptomyces durhamensis*)와의 비교 시, 높은 셀룰레이즈, 자일란네이즈 및 과산화 효소 활성을 나타내었다. 이러한 실험 결과를 토대로, 89개의 분리 균주 중 리그노셀룰로오스 분해 연구의 최종 후보로 *Bacillus* sp. 275를 선정하였다.

세 번째로, *Bacillus* sp. 275의 유전체 서열을 확보하고 이를 통한 유전체 분석을 연구 하였다. *Bacillus* sp. 275의 유전체에서 리그노셀룰로오스의 분해에 관여하는 많은 유전자(β-glucanase, glucohydrolase, glucosidase, 1,4-β-xylosidase, arabinoxylan arabinofuranohydrolase, glucuronoxylanase, dye decolorizing peroxidase 및 laccase)가 발견되었다. 또한, 전분, 만난, 갈락토사이드 및 아라비난의 분해와 관련된 유전자도 발견되었다.

마지막으로, *Bacillus* sp. 275의 리그노셀룰로오스 분해 특성을 연구하였다. *Bacillus* sp. 275에서 분비된 세포 외 단백질에 의해 셀룰로오스와 자일란의 분해 산물(cellobiose와 cellotriose, 그리고, xylose, xylobiose, xylotriose, xylotetraose, xylopentaose)이 관찰되었다. 또한, 11일 동안 *Bacillus* sp. 275에 의해 여과지 분해가 활발하게 일어나는 것을 관찰하였다. 리그닌 이용에 관해서는 리그닌이 첨가된 배지에서의 세포 성장이 리그닌이 없는 배지에 비해 78% 증가하였다. 또한, 리그닌이 첨가된 경우, lag phase가 12시간에서 6시간으로 단축됨을 확인하였다.

본 연구에서 샘플링 위치 및 분리 배지와 같은 분리 조건이 리그노

셀룰로오스 분해 박테리아를 효율적으로 분리하는 중요한 요인임을 확인할 수 있었다. 분리 균주 *Bacillus* sp. 275는 세포 외로 생산된 효소에 의하여 셀룰로오스, 자일란, 리그닌 분해 활성을 보였다. 또한, *Bacillus* sp. 275는 셀룰로오스, 자일란, 리그닌 뿐만 아니라, 잠재적으로 리그노셀룰로오스 바이오매스의 광범위한 다른 종류의 다당류 역시 분해 할 수 있음을 확인하였다. 리그노셀룰로오스의 주요 3가지 구성 성분 (즉, 셀룰로오스, 헤미셀룰로오스, 리그닌)을 모두 사용하는 박테리아에 대한 보고가 흔치 않음을 고려하면, *Bacillus* sp. 275는 리그노셀룰로오스 바이오매스를 분해하거나 이용할 수 있는 유망한 미생물 자원 중 하나라 할 수 있을 것이다.

주요어: 박테리아 분리, 리그노셀룰로오스 분해 박테리아, 리그노셀룰로오스 분해, *Bacillus* sp. 275, 유전체 서열 분석

학 번: 2008-30288